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APPLICATION FOR LETTERS PATENT

for

COMPLEMENTING CELL LINES

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TITLE OF THE INVENTION
COMPLEMENTING CELL LINES

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application is a continuation-in-part of application Serial No. 09/713,678, filed November 15, 2000, pending.

TECHNICAL FIELD

[0002] The invention relates to the field of biotechnology generally and, more specifically, to adenoviral-based complementing cell lines.

BACKGROUND OF THE INVENTION

[0003] Typically, vector and packaging cells have to be adapted to one another so that they have all the necessary elements, but they do not have overlapping elements that lead to replication-competent virus by recombination. Therefore, the sequences necessary for proper transcription of the packaging construct may be heterologous regulatory sequences derived from, for example, other human adenovirus (Ad) serotypes, nonhuman adenoviruses, other viruses like, but not limited to, SV40, hepatitis B virus (HBV), Rous Sarcoma Virus (RSV), cytomegalovirus (CMV), etc. or from higher eukaryotes such as mammals. In general, these sequences include a promoter, enhancer and poly-adenylation sequences.

[0004] PER.C6 (ECACC deposit number 96022940) is an example of a cell line devoid of sequence overlap between the packaging construct and the adenoviral vector (Fallaux *et al.*, 1998). Recombinant viruses based on subgroup C adenoviruses, such as Ad5 and Ad2, can be propagated efficiently on these packaging cells. Generation and propagation of adenoviruses from other serotypes, like subgroup B viruses, has proven to be more difficult on PER.C6 cells. However, as described in European patent application 00201738.2, recombinant viruses based on subgroup B virus Ad35 can be made by co-transfection of an expression construct containing the Ad35 early region-1 sequences (Ad35-E1). Furthermore, Ad35-based viruses that are deleted for E1A sequences were shown to replicate efficiently on PER.C6 cells. Thus, the E1A proteins of

Ad5 complement Ad35-E1A functions, whereas, at least part of the E1B functions of Ad35 are necessary. This serotype specificity in E1B functions was recently also described for Ad7 recombinant viruses. In an attempt to generate recombinant adenoviruses derived from subgroup B virus Ad7, Abrahamsen *et al.* (1997) were not able to generate E1-deleted viruses on 293 cells without contamination of wild-type (wt) Ad7. Viruses that were picked after plaque purification on 293-ORF6 cells (Brough *et al.*, 1996) were shown to have incorporated Ad7-E1B sequences by nonhomologous recombination. Thus, efficient propagation of Ad7 recombinant viruses proved possible only in the presence of Ad7-E1B expression and Ad5-E4-ORF6 expression. The E1B proteins are known to interact with cellular, as well as viral, proteins (Bridge *et al.*, 1993; White, 1995). Possibly, the complex formed between the E1B-55K protein and E4-ORF6 which is necessary to increase mRNA export of viral proteins and to inhibit export of most cellular mRNAs, is critical and in some way serotype-specific.

DESCRIPTION OF THE INVENTION

[0005] The present invention provides new packaging cell lines capable of complementing recombinant adenoviruses based on serotypes other than subgroup C viruses, such as serotypes from subgroup B like adenovirus type 35.

[0006] In one aspect, the invention provides packaging cell lines capable of complementing recombinant adenovirus based on a serotype of subgroup B, preferably of serotype 35. With the terms "based on or derived from an adenovirus" is meant that it utilizes nucleic acid corresponding to nucleic acid found in the serotype. The utilized nucleic acid may be derived by PCR cloning or other methods known in the art.

[0007] In one aspect, the new packaging cells are derived from primary, diploid human cells such as, but not limited to, primary human retinoblasts, primary human embryonic kidney cells or primary human amniocytes. Transfection of primary cells or derivatives thereof with the adenovirus E1A gene alone can induce unlimited proliferation (immortalization), but does not result in complete transformation. However, expression of E1A in most cases results in induction of programmed cell death (apoptosis), and occasionally immortalization is obtained (Jochemsen *et al.*, 1987). Co-expression of the E1B gene is required to prevent induction of apoptosis and for complete

morphological transformation to occur (reviewed in White, 1995). Therefore, in one aspect of the invention, primary human cells or derivatives thereof are transformed by expression of adenovirus E1 proteins of a subgroup other than subgroup C, preferably subgroup B, more preferably adenovirus type 35. The combined activity of the E1A and E1B proteins establishes indefinite growth of the cells and enables complementation of recombinant adenoviruses.

[0008] The complete morphological transformation of primary cells by adenovirus E1 genes is the result of the combined activities of the proteins encoded by the E1A and E1B regions. The roles of the different E1 proteins in lytic infection and in transformation have been studied extensively (reviewed in Zantema and van der Eb, 1995; White, 1995, 1996). The adenovirus E1A proteins are essential for transformation of primary cells. The E1A proteins exert this effect through direct interaction with a number of cellular proteins that are involved in regulation of transcription. These include the pRB family of proteins, p300/CBP and TATA binding protein. In addition to this E1A increases the level of p53 protein in the cells. In the absence of adenovirus E1B activity the rise in p53 levels leads to the induction of apoptosis. Both proteins encoded by the E1B region counteract the induction of apoptosis although by different mechanisms. E1B-21K seems to counteract apoptosis in a manner similar to Bcl-2 via interaction with the effector proteins downstream in the apoptosis pathway (Han *et al.*, 1996), whereas E1B-55K functions through direct interaction with p53. Importantly, the molecular mechanism by which the E1B-55K proteins of Ad2 and 5 (subgroup C) and Ad12 (subgroup A) function in the ability to neutralise p53 may differ. Whereas Ad5 E1B-55K binds p53 strongly and the complex localises to the cytoplasm, Ad12 E1B-55K binds p53 weakly and both proteins are localised in the nucleus (Zantema *et al.*, 1985; Grand *et al.*, 1999). Both proteins, however, inhibit the transactivation of other genes by p53 (Yew and Berk, 1992).

[0009] In rodent cells, the activity of E1A together with either E1B-21K or 55K is sufficient for full transformation although expression of both E1B proteins together is twice as efficient (Rao *et al.*, 1992). In human cells however, the activity of the E1B-55K protein seems to be more important given the observation that E1B-55K is indispensable for the establishment of transformed cells (Gallimore, 1986).

[0010] Example 6 hereof describes the generation of pIG270. In this construct the Ad35-E1 genes are expressed from the hPGK promoter and transcription is terminated by the HBVpA. The hPGK promoter constitutes a HincII-EcoRI fragment of the promoter sequence described by Singer-Sam *et al.* (1984). The HBVpA is located in a BamHI-BglIII fragment of the Hepatitis B virus genome (Simonsen and Levinson, 1983; *see also* Genbank HBV-AF090841). As mentioned before, the promoter and polyadenylation sequences of the E1 expression constructs described in this invention may be derived from other sources without departing from the invention. Also, other functional fragments of the hPGK and HBVpA sequences mentioned above may be used.

[0011] The functionality of pIG270 was shown by transformation of primary Baby Rat Kidney cells (BRK). Comparison with an equivalent Ad5-E1 expression construct taught that Ad35-E1 genes were less efficient in transforming these cells. The same has been found for the E1 genes of Ad12 (Bernards *et al.*, 1982).

[0012] It is unclear which E1 protein(s) determine(s) the difference in transformation efficiency of E1 sequences observed for adenoviruses from different subgroups. In the case of Ad12, transfection studies with chimeric E1A/E1B genes suggested that the efficiency of transformation of BRK cells was determined by the E1A proteins (Bernards *et al.*, 1982). The E1B-55K protein is shown *infra* to contain serotype-specific functions necessary for complementation of E1-deleted adenoviruses. If these functions are related to the regulation of mRNA distribution or another late viral function, it is unlikely that these are involved in the transformation efficiency.

[0013] Analysis of functional domains in the Ad2 or Ad5 E1B-55K proteins using insertion mutants have revealed that functions related to viral replication, late protein synthesis and host protein shut-off are not confined to specific domains but are distributed along the protein (Yew *et al.*, 1990). Using the same set of mutants, the domains important for interaction with p53 and E4-Orf6 were found to be more restricted. In addition to one common binding region (amino acids 262 to 326), p53 binding was affected by mutations at aa 180 and E4-Orf6 binding was affected by mutations at aa 143 (Yew and Berk, 1992; Rubenwolf *et al.*, 1997).

[0014] Altogether these results indicate that it is difficult to separate the E1B-55K functions related to transformation (p53 binding) and late protein synthesis (Orf6 binding).

[0015] The invention discloses new E1 constructs that combine the high efficiency of transformation of one serotype with the serotype-specific complementation function of another serotype. These new constructs are used to transform primary human embryonic retinoblast cells and human amniocytes.

[0016] In another aspect of the invention, the transforming E1 sequences are derived from different serotypes. As disclosed in European Patent application 00201738.2, Ad35E1 sequences are capable of transforming Baby Rat Kidney (BRK) cells, albeit with a lower efficiency than that seen with Ad5-E1 sequences. This was also observed for E1 sequences from Ad12 (Bernards *et al.*, 1982). Therefore, in this aspect of the invention, primary diploid human cells or derivatives thereof are transformed with chimeric E1 construct that consists of part of the E1 sequences of a serotype that enables efficient transformation of primary human cells or derivatives thereof and part of the E1 sequences of another serotype which E1 sequences provide the serotype-specific E1B function(s) that enable(s) efficient propagation of E1-deleted viruses of that serotype. In a preferred embodiment of this aspect of the invention, the E1A region is derived from a subgroup C adenovirus like, but not limited to, Ad5, and the E1B coding sequences are derived from an alternative adenovirus, more particularly from an adenovirus of subgroup B, even more particularly from adenovirus type 35. E1B-21K coding sequences may also be chimeric comprising both subgroup C and subgroup B coding sequences. Preferably, all or most of E1B-21K comprises subgroup C coding sequences. In a more preferred embodiment, the E1A coding sequences and the E1B-21K coding sequences are derived from a subgroup C adenovirus, like, but not limited to, Ad5. In one embodiment the cell further comprises E1B-55k coding sequences that are, preferably, as far as not overlapping with the 21K coding sequences-derived from an adenovirus of subgroup B, more particularly from adenovirus type 35. In an even more preferred embodiment, all E1 coding sequences are derived from a subgroup C adenovirus, like but not limited to Ad5, except for at least the part of the E1B-55K coding sequences that are necessary for serotype-specific complementation of an alternative adenovirus subgroup, more

particularly adenovirus subgroup B, even more particular adenovirus type 35. The invention also provides a packaging cell line wherein the primary, diploid human cells or derivatives thereof have been transformed with a chimeric adenovirus E1 construct comprising part of a first adenovirus E1 coding sequence of a first adenovirus serotype that enables efficient transformation of primary human cells and derivatives thereof; and part of a second adenovirus E1 coding sequence of a second adenovirus serotype, wherein the second adenovirus E1 coding sequence provides the serotype-specific adenovirus E1B function(s) that enable(s) efficient propagation of recombinant adenovirus E1-deleted viruses of the second adenovirus serotype. Preferably, the first adenovirus serotype is a subgroup C adenovirus and the second adenovirus serotype is a subgroup B adenovirus, more particular adenovirus type 35. In one embodiment the packaging cell line of the invention comprises bovine adenovirus E1B-55k. Such a bovine E1B-55k expressing cell line is particularly suited for obtaining high yields of a complemented bovine recombinant adenovirus.

[0017] The primary diploid human cells or derivatives thereof are transformed by adenovirus E1 sequences, either operatively linked on one DNA molecule or located on two separate DNA molecules. In the latter case, one DNA molecule carries at least part of the E1 sequences of the serotype-enabling efficient transformation and the second DNA molecule carries at least part of the sequences necessary for serotype-specific complementation. Also provided is a hybrid construct including E1-sequences of the serotype enabling efficient transformation and E1 sequences of another serotype necessary for serotype-specific complementation. The sequences providing serotype specific complementation may of course also contain further activities contributing to transformation. Preferably, the sequences enabling efficient transformation comprise E1A. Preferably, the sequences and the sequences necessary for serotype specific complementation preferably comprise E1B sequences. More preferably, the sequences enabling efficient transforming comprise E1A and E1B-21K sequences and the sequences necessary for serotype specific complementation comprise E1B-55K sequences. Also provided are cells transformed by such hybrid construct. Such cells can favorably be used for the propagation of recombinant E1 deleted adenovirus of another serotype. Of course, it is also possible to provide both functions of E1 sequences on separate constructs. In all

aspects, the sequences are operatively linked to regulatory sequences enabling transcription and translation of the encoded proteins. Preferably, a packaging cell of the invention further comprises a DNA encoding at least E4-orf6 of an adenovirus of subgroup B, preferably adenovirus serotype 35. Preferably, the E4-orf6 is derived from the another serotype. Preferably, the cell comprises E1B-55K and E4-orf6 of the same serotype as the recombinant vector to be propagated/complemented or otherwise produced.

[0018] In another aspect of the invention, new packaging cells are described that are derived from PER.C6 (ECACC deposit number 96022940; Fallaux *et al.*, 1998) and contain Ad35-E1 sequences integrated into their genome. These Ad35-E1 sequences are present in a functional expression cassette, but preferably do not contain sequences overlapping with sequences present in the recombinant viral vector. Preferably, the functional expression cassette consists of a heterologous promoter and poly-adenylation signal functionally linked to Ad35-E1 sequences. More specifically, the Ad35-E1 coding sequences are functionally linked to the human phosphoglycerate gene promoter (hPGK) and hepatitis B virus poly-adenylation signal (HBV-pA). Preferably, Ad35-E1 coding sequences comprise the coding regions of the E1A proteins and the E1B promoter sequences linked to E1B coding sequences up to and including the stop codon of the E1B 55K protein. More preferably, the Ad35-E1 sequences comprise nucleotide 468 to nucleotide 3400 of the Ad35 wt sequence. To be able to select for transfected cells, a dominant selection marker like, but not limited to, the *neo^r* gene has to be incorporated on the expression vector or the Ad35 expression vector is cotransfected with a separate expression vector mediating expression of the selection marker. In both cases, the selection marker becomes integrated in the cellular genome. Other Ad5-E1 transformed cell lines like 293 (Graham *et al.*, 1977) and 911 (Fallaux *et al.*, 1996) or established human cell lines like A549 cells may be used without departing from the present invention.

[0019] In another aspect of the invention, PER.C6-derived cells are described that express functional Ad35-E1B sequences. In one embodiment, the Ad35-E1B coding sequences are driven by the E1B promoter and terminated by a heterologous poly-adenylation signal like, but not limited to, the HBVpA. In a preferred embodiment, the

Ad35-E1B coding sequences are driven by a heterologous promoter like, but not limited to, the hPGK promoter or Elongation Factor-1 α (EF-1 α) promoter and terminated by a heterologous pA signal like, but not limited to, the HBVpA. These Ad35-E1B sequences preferably comprise the coding regions of the E1B-21K and the E1B-55K proteins located between nucleotides 1611 and 3400 of the wild-type (wt) Ad35 sequence. More preferably, the Ad35-E1B sequences comprise nucleotides 1550 to 3400 of the wt Ad35 sequence. In an even more preferred embodiment, the E1B sequences comprise the coding sequences of the E1B-55K gene located between nucleotides 1916 and 3400 of the wt Ad35 sequence. In an even more preferred embodiment a packaging cell line or a cell line of the invention lacks a functional coding sequence for E1B 21k. Such cell lines, in general, produce significantly more recombinant adenovirus than E1B 21K positive cell lines.

[0020] The invention further provides a method for complementing a recombinant adenovirus comprising providing a packaging cell line or a cell line according to the invention, with the recombinant adenovirus and culturing the cell to allow for complementation. In a preferred embodiment the method further comprises harvesting complemented recombinant adenovirus. Preferably, the recombinant adenovirus is derived from adenovirus subgroup B. More preferably, the recombinant adenovirus is derived from adenovirus serotype 35.

[0021] In another aspect, the invention provides a recombinant adenovirus obtained by a method of the invention or with a packaging cell of the invention. Such an adenovirus can be obtained essentially free from contaminating wild type adenovirus, or replication competent adenovirus. Such recombinant adenovirus preparations are very suited for administration of therapeutic sequences to somatic tissues *in vivo* in for instance a gene therapeutic setting. Preferred are recombinant adenoviruses comprising a deletion of nucleic acid encoding at least one E1-region protein. Preferably, such adenovirus further comprises a deletion of nucleic acid encoding at least one E3-region protein. Preferably, such adenovirus further comprises a deletion of nucleic acid encoding at least one E4-region protein. Preferably, such adenovirus further comprises a deletion of nucleic acid encoding at least E4-Orf6 protein. For this reason, the invention also

provides the use of a recombinant adenovirus of the invention for the preparation of a medicament.

[0022] With the term E1B-55K protein as used herein, is meant the protein encoded by the E1B-region in an adenovirus serotype having a similar function in the serotype as provided by the E1B-55K protein Ad5.

[0023] With the term E1B-21K protein as used herein, is meant the protein enclosed by the E1B-region in an adenovirus serotype having a similar function in the serotype as provided by the E1B-19K protein of Ad5. The same terminology applies for the sequences encoding these proteins. When referring to Ad35-E1 sequences from a specified nucleotide to nucleotide 3400 is meant 'up to and including nucleotide 3400'.

[0024] Cell lines subject of this invention are useful for, among other things, the production of recombinant adenoviruses designed for gene therapy and vaccination. The cell lines, being derived from cells of human origin, are also useful for the production of human recombinant therapeutic proteins like, but not limited to human growth factors, human antibodies. In addition the cell lines are useful for the production of human viruses other than adenovirus like, but not limited to, influenza virus, herpes simplex virus, rotavirus, measles virus.

[0025] A preferred derivative of primary, diploid human cells is the PER.C6 cell line (ECACC deposit number 960022940).

[0026] It is within the skills of the artisan to provide for proteins having a similar function in kind as the adenovirus E1 protein referred to in this document. For instance a functional part may be provided and/or a derivative may be provided with a similar function in kind, not necessarily in amount.

[0027] Such parts and derivatives are considered to be part of the invention, in as far as similar transforming/complementing and/or serotype specificity function is provided in kind, not necessarily in amount.

BRIEF DESCRIPTION OF THE FIGURES

[0028] FIG. 1: Bar graph showing the percentage of serum samples positive for neutralization for each human wt adenovirus tested (*see*, Example 1 for description of the neutralization assay).

[0029] FIG. 2: Graph showing absence of correlation between the VP/CCID50 ratio and the percentage of neutralization.

[0030] FIG. 3: Bar graph presenting the percentage sera samples that show neutralizing activity to a selection of adenovirus serotypes. Sera were derived from healthy volunteers from Belgium and the UK.

[0031] FIG. 4: Bar graph presenting the percentage sera samples that show neutralizing activity to adenovirus serotypes 5, 11, 26, 34, 35, 48 and 49. Sera were derived from five different locations in Europe and the United States.

[0032] FIG. 5: Sequence of human adenovirus type 35.

[0033] FIG. 6: Map of pAdApt35IP1.

[0034] FIG. 7: Schematic representation of the steps undertaken to construct pWE.Ad35.pIX-rITR.

[0035] FIG. 8: Map of pWE.Ad35.pIX-rITR.

[0036] FIG. 9: Map of pRSV.Ad35-E1.

[0037] FIG. 10: Map of pPGKneopA

[0038] FIG. 11: Map of pRSV-Pneo.

[0039] FIG. 12: Map of pRSVhbv.Neo.

[0040] FIG. 13: Map of pIG.E1A.E1B.

[0041] FIG. 14: Map of pIG135.

[0042] FIG. 15: Map of pIG270.

[0043] FIG. 16: Map of pBr.Ad35.leftITR-pIX.

[0044] FIG. 17: Map of pBr.Ad35.leftITR-pIXdE1A

[0045] FIG. 18: Map of pBr.Ad35.d21K

[0046] FIG. 19: Map of pBr.Ad35.d55K1

[0047] FIG. 20: Map of pBr.Ad35dSM

[0048] FIG. 21: Schematic representation of Ad35-E1A/E1B deletion constructs.

[0049] FIG. 22: Map of pIG.35BL.

[0050] FIG. 23: Map of pRSVneo4.

[0051] FIG. 24: Map of pIG35Bneo.

[0052] FIG. 25: Map of pIG35.55K

- [0053] FIG. 26: Map of pIG535
[0054] FIG. 27: Map of pIG635
[0055] FIG. 28: Map of pIG735
[0056] FIG 29: Map of pCC271
[0057] FIG 30: Map of pCC535s
[0058] FIG 31: Map of pCR535E1B
[0059] FIG 32: Map of pCC2155s
[0060] FIG 33: Map of pCC536s
[0061] FIG 34: Map of pIG536
[0062] FIG 35: Map of pBr.Ad35.PRn
[0063] FIG 36: Map of pBr.Ad35.PRnΔE3
[0064] FIG 37: Map of pWE.Ad35.pIX-rITRAE3
[0065] FIG 38: Alignment of E1B-21K (A) and E1B-55K (B) amino acid sequences in pCC536s with wtAd5 and wtAd35 sequences.

DETAILED DESCRIPTION OF THE INVENTION

[0066] The invention is further explained by the use of the following illustrative examples.

EXAMPLES

Example 1

A high throughput assay for the detection of neutralizing activity in human serum

[0067] To enable screening of a large amount of human sera for the presence of neutralizing antibodies against all adenovirus serotypes, an automated 96-wells assay was developed.

Human sera

[0068] A panel of 100 individuals was selected. Volunteers (50% male, 50% female) were healthy individuals between ages 20 and 60 years old with no restriction for race. All volunteers signed an informed consent form. People professionally involved in adenovirus research were excluded.

[0069] Approximately 60 ml blood was drawn in dry tubes. Within two hours after sampling, the blood was centrifuged at 2500 rpm for 10 minutes. Approximately 30 ml serum was transferred to polypropylene tubes and stored frozen at -20°C until further use.

[0070] Serum was thawed and heat-inactivated at 56°C for 10 minutes and then aliquoted to prevent repeated cycles of freeze/thawing. Part was used to make five steps of twofold dilutions in medium (DMEM, Gibco BRL) in a quantity large enough to fill out approximately 70 96-well plates. Aliquots of undiluted and diluted sera were pipetted in deep well plates (96-well format) and using a programmed platemate dispensed in 100 µl aliquots into 96-well plates. The plates were loaded with eight different sera in duplo (100 µl/well) according to the scheme below:

S1/2	S1/4	S1/8	S1/1 6	S1/3 2	S5/2	S5/4	S5/8	S5/1 6	S5/3 2	-	-
S1/2	S1/4	S1/8	S1/1 6	S1/3 2	S5/2	S5/4	S5/8	S5/1 6	S5/3 2	-	-
S2/2	S2/4	S2/8	S2/1 6	S2/3 2	S6/2	S6/4	S6/8	S6/1 6	S6/3 2	-	-
S2/2	S2/4	S2/8	S2/1 6	S2/3 2	S6/2	S6/4	S6/8	S6/1 6	S6/3 2	-	-
S3/2	S3/4	S3/8	S3/1 6	S3/3 2	S7/2	S7/4	S7/8	S7/1 6	S7/3 2	-	-
S3/2	S3/4	S3/8	S3/1 6	S3/3 2	S7/2	S7/4	S7/8	S7/1 6	S7/3 2	-	-
S4/2	S4/4	S3/8	S3/1 6	S3/3 2	S8/2	S8/4	S8/8	S8/1 6	S8/3 2	-	-
S4/2	S4/4	S3/8	S3/1 6	S3/3 2	S8/2	S8/4	S8/8	S8/1 6	S8/3 2	-	-

Where S1/2 to S8/2 in columns 1 and 6 represent 1 X diluted sera and Sx/4, Sx/8, Sx/16 and Sx/32 the twofold serial dilutions. The last plates also contained four wells filled with 100 µl fetal calf serum as a negative control. Plates were kept at -20°C until further use.

Preparation of human adenovirus stocks

[0071] Prototypes of all known human adenoviruses were inoculated on T25 flasks seeded with PER.C6 cells (Fallaux *et al.*, 1998) and harvested upon full CPE. After freeze/thawing, 1-2 ml of the crude lysates were used to inoculate a T80 flask with PER.C6 and virus was harvested at full CPE. The timeframe between inoculation and occurrence of CPE, as well as the amount of virus needed to re-infect a new culture,

differed between serotypes. Adenovirus stocks were prepared by freeze/thawing and used to inoculate 3-4 T175 cm² three-layer flasks with PER.C6 cells. Upon occurrence of CPE, cells were harvested by tapping the flask, pelleted and virus was isolated and purified by a two-step CsCl gradient as follows. Cell pellets were dissolved in 50 ml 10 mM NaPO₄ buffer (pH 7.2) and frozen at -20°C. After thawing at 37°C, 5.6 ml sodium deoxycholate (5% w/v) was added. The solution was mixed gently and incubated for 5-15 minutes at 37°C to completely lyse the cells. After homogenizing the solution, 1875 µl 1M MgCl₂ was added. After the addition of 375 µl DNase (10 mg/ml), the solution was incubated for 30 minutes at 37°C. Cell debris was removed by centrifugation at 1880xg for 30 minutes at RT without brake. The supernatant was subsequently purified from proteins by extraction with FREON (3x). The cleared supernatant was loaded on a 1M Tris/HCl buffered cesium chloride block gradient (range: 1.2/1.4 g/ml) and centrifuged at 21000 rpm for 2.5 hours at 10°C. The virus band is isolated after which a second purification using a 1M Tris/HCl buffered continuous gradient of 1.33 g/ml of cesium chloride was performed. The virus was then centrifuged for 17 hours at 55000 rpm at 10°C. The virus band is isolated and sucrose (50 % w/v) is added to a final concentration of 1%. Excess cesium chloride is removed by dialysis (three times 1 hr at RT) in dialysis slides (Slide-a-lizer, cut off 10000 kDa, Pierce, USA) against 1.5 liter PBS supplemented with CaCl₂ (0.9 mM), MgCl₂ (0.5mM) and an increasing concentration of sucrose (1, 2, 5%). After dialysis, the virus is removed from the slide-a-lizer after which it is aliquoted in portions of 25 and 100 µl upon which the virus is stored at -85°C.

[0072] To determine the number of virus particles per milliliter, 50 µl of the virus batch is run on a high-pressure liquid chromatograph (HPLC) as described by Shabram et al (1997). Viruses were eluted using a NaCl gradient ranging from 0 to 600 mM. As depicted in table I, the NaCl concentration by which the viruses were eluted differed significantly among serotypes.

[0073] Most human adenoviruses replicated well on PER.C6 cells with a few exceptions. Adenovirus type 8 and 40 were grown on 911-E4 cells (He *et al.*, 1998). Purified stocks contained between 5x10¹⁰ and 5x10¹² virus particles/ml (VP/ml; see table I).

Titration of purified human adenovirus stocks

[0074] Adenoviruses were titrated on PER.C6 cells to determine the amount of virus necessary to obtain full CPE in five days, the length of the neutralization assay. Hereto, 100 µl medium was dispensed into each well of 96-well plates. 25 µl of adenovirus stocks pre-diluted 10^4 , 10^5 , 10^6 or 10^7 times were added to column 2 of a 96-well plate and mixed by pipetting up and down 10 times. Then 25 µl was brought from column 2 to column 3 and again mixed. This was repeated until column 11, after which 25 µl from column 11 was discarded. This way, serial dilutions in steps of 5 were obtained starting off from a pre-diluted stock. Then 3×10^4 PER.C6 cells (ECACC deposit number 96022940) were added in a 100 µl volume and the plates were incubated at 37 °C, 5% CO₂ for five or six days. CPE was monitored microscopically. The method of Reed and Muensch was used to calculate the cell culture-inhibiting dose 50% (CCID50).

[0075] In parallel, identical plates were set up that were analyzed using the MTT assay (Promega). In this assay, living cells are quantified by colorimetric staining. Hereto, 20 µl MTT (7.5 mgr/ml in PBS) was added to the wells and incubated at 37 °C, 5% CO₂ for two hours. The supernatant was removed and 100 µl of a 20:1 isopropanol/triton-X100 solution was added to the wells. The plates were put on a 96-well shaker for 3-5 minutes to solubilize the precipitated staining. Absorbance was measured at 540 nm and at 690 nm (background). By this assay, wells with proceeding CPE or full CPE can be distinguished.

Neutralization assay

[0076] 96-well plates with diluted human serum samples were thawed at 37 °C, 5% CO₂. Adenovirus stocks diluted to 200 CCID50 per 50 µl were prepared and 50 µl aliquots were added to columns 1-11 of the plates with serum. Plates were incubated for 1 hour at 37°C, 5% CO₂. Then, 50 µl PER.C6 cells at 6×10^5 /ml were dispensed in all wells and incubated for 1 day at 37 °C, 5% CO₂. Supernatant was removed using fresh pipette tips for each row and 200 µl fresh medium was added to all wells to avoid toxic effects of the serum. Plates were incubated for another 4 days at 37 °C, 5% CO₂. In addition, parallel control plates were set up *in duplo*, with diluted positive control sera generated in rabbits and specific for each serotype to be tested in rows A and B and with

negative control serum (FCS) in rows C and D. Also, in each of the rows E-H, a titration was performed as described above with steps of five times dilutions starting with 200 CCID50 of each virus to be tested. On day 5, one of the control plates was analyzed microscopically and with the MTT assay. The experimental titer was calculated from the control titration plate observed microscopically. If CPE was found to be complete, *i.e.*, the first dilution in the control titration experiment analyzed by MTT shows clear cell death, all assay plates were processed. If not, the assay was allowed to proceed for one or more days until full CPE was apparent, after which all plates were processed. In most cases, the assay was terminated at day 5. For Ad1, 5, 33, 39, 42 and 43 the assay was left for six days and for Ad2 for eight days.

[0077] A serum sample is regarded as “non-neutralizing” when, at the highest serum concentration, a maximum protection of 40% is seen compared to controls without serum.

[0078] The results of the analysis of 44 prototype adenoviruses against serum from 100 healthy volunteers are shown in FIG. 1. As expected, the percentage of serum samples that contained neutralizing antibodies to Ad2 and Ad5 was very high. This was also true for most of the lower numbered adenoviruses. Surprisingly, none of the serum samples contained neutralizing antibodies to Ad35. Also, the number of individuals with neutralizing antibody titers to the serotypes 26, 34 and 48 was very low. Therefore, recombinant E1-deleted adenoviruses based on Ad35 or one of the other above-mentioned serotypes have an important advantage compared to recombinant vectors based on Ad5 with respect to clearance of the viruses by neutralizing antibodies.

[0079] Also, Ad5-based vectors that have parts of the capsid proteins involved in immunogenic response of the host replaced by the corresponding parts of the capsid proteins of Ad35 or one of the other serotypes will be less, or even not, neutralized by the vast majority of human sera.

[0080] As can be seen in Table I, the VP/CCID50 ratio calculated from the virus particles per ml and the CCID50 obtained for each virus in the experiments was highly variable and ranged from 0.4 to 5 log. This is probably caused by different infection efficiencies of PER.C6 cells and by differences in replication efficiency of the viruses. Furthermore, differences in batch qualities may play a role. A high VP/CCID50

ratio means that more viruses were put in the wells to obtain CPE in 5 days. As a consequence, the outcome of the neutralization study might be biased since more inactive virus particles could shield the antibodies. To check whether this phenomenon had taken place, the VP/CCID50 ratio was plotted against the percentage of serum samples found positive in the assay (FIG. 2). The graph clearly shows that there is no negative correlation between the amount of viruses in the assay and neutralization in serum.

Example 2

The prevalence of neutralizing activity (NA) to Ad35 is low in human sera from different geographic locations

[0081] In Example 1, the analysis of neutralizing activity ("NA") in human sera from one location in Belgium was described. Strikingly, of a panel of 44 adenovirus serotypes tested, one serotype, Ad35, was not neutralized in any of the 100 sera assayed. In addition, a few serotypes, Ad26, Ad34 and Ad48 were found to be neutralized in 8%, or less, of the sera tested. This analysis was further extended to other serotypes of adenovirus not previously tested and, using a selection of serotypes from the first screen, was also extended to sera from different geographic locations.

[0082] Hereto, adenoviruses were propagated, purified and tested for neutralization in the CPE-inhibition assay as described in Example 1. Using the sera from the same batch as in Example 1, adenovirus serotypes 7B, 11, 14, 18 and 44/1876 were tested for neutralization. These viruses were found to be neutralized in, respectively, 59, 13, 30, 98 and 54 % of the sera. Thus, of this series, Ad11 is neutralized with a relatively low frequency.

[0083] Since it is known that the frequency of isolation of adenovirus serotypes from human tissue, as well as the prevalence of NA to adenovirus serotypes, may differ on different geographic locations, we further tested a selection of the adenovirus serotypes against sera from different places. Human sera were obtained from two additional places in Europe (Bristol, UK and Leiden, NL) and from two places in the United States (Stanford, CA and Great Neck, NY). Adenoviruses that were found to be neutralized in 20% or less of the sera in the first screen, as well as Ad2, Ad5, Ad27, Ad30, Ad38, Ad43, were tested for neutralization in sera from the UK. The results of

these experiments are presented in FIG. 3. Adenovirus serotypes 2 and 5 were again neutralized in a high percentage of human sera. Furthermore, some of the serotypes that were neutralized in a low percentage of sera in the first screen are neutralized in a higher percentage of sera from the UK, for example, Ad26 (7% vs. 30%), Ad28 (13% vs. 50%), Ad34 (5% vs. 27%) and Ad48 (8% vs. 32%). Neutralizing activity against Ad11 and Ad49 that were found in a relatively low percentage of sera in the first screen, are found in an even lower percentage of sera in this second screen (13% vs. 5% and 20% vs. 11%, respectively). Serotype Ad35 that was not neutralized in any of the sera in the first screen, was now found to be neutralized in a low percentage (8%) of sera from the UK. The prevalence of NA in human sera from the UK is the lowest to serotypes Ad11 and Ad35.

[0084] For further analysis, sera was obtained from two locations in the US (Stanford, CA and Great Neck, NY) and from The Netherlands (Leiden). FIG. 4 presents an overview of data obtained with these sera and the previous data. Not all viruses were tested in all sera, except for Ad5, Ad11 and Ad35. The overall conclusion from this comprehensive screen of human sera is that the prevalence of neutralizing activity to Ad35 is the lowest of all serotypes throughout the western countries: on average 7% of the human sera contain neutralizing activity (5 different locations). Another B-group adenovirus, Ad11 is also neutralized in a low percentage of human sera (average 11% in sera from 5 different locations). Adenovirus type 5 is neutralized in 56% of the human sera obtained from 5 different locations. Although not tested in all sera, D-group serotype 49 is also neutralized with relatively low frequency in samples from Europe and from one location of the US (average 14%).

[0085] In the herein described neutralization experiments, a serum is judged non-neutralizing when, in the well with the highest serum concentration, the maximum protection of CPE is 40% compared to the controls without serum. The protection is calculated as follows:

$$1\% \text{ protection} = \frac{\text{OD corresponding well} - \text{OD virus control}}{\text{OD non-infected control} - \text{OD virus control}} \times 100 \%$$

[0086] As described in Example 1, the serum is plated in five different dilutions ranging from 4x to 64x diluted. Therefore, it is possible to distinguish between low titers (*i.e.*, neutralization only in the highest serum concentrations) and high titers of NA (*i.e.*, also neutralization in wells with the lowest serum concentration). Of the human sera used in our screen that were found to contain neutralizing activity to Ad5, 70% turned out to have high titers, whereas, of the sera that contained NA to Ad35, only 15% had high titers. Of the sera that were positive for NA to Ad11, only 8% had high titers. For Ad49, this was 5%. Therefore, not only is the frequency of NA to Ad35, Ad11 and Ad49 much lower as compared to Ad5, but of the sera that do contain NA to these viruses, the vast majority have low titers. Adenoviral vectors based on Ad11, Ad35 or Ad49 have, therefore, a clear advantage over Ad5-based vectors when used as gene therapy vehicles or vaccination vectors *in vivo* or in any application where infection efficiency is hampered by neutralizing activity.

[0087] In the following examples, the construction of a vector system for the generation of safe, RCA-free Ad35-based vectors is described.

Example 3

Sequence of the human adenovirus type 35

[0088] Ad35 viruses were propagated on PER.C6 cells and DNA was isolated as follows: To 100 μ l of virus stock (Ad35: 3.26×10^{12} VP/ml), 10 μ l 10X DNase buffer (130 mM Tris-HCl pH7.5; 1,2 M CaCl_2 ; 50mM MgCl_2) was added. After addition of 10 μ l 10mgr/ml DNase I (Roche Diagnostics), the mixture was incubated for 1 hr. at 37°C. Following addition of 2.5 μ l 0.5M EDTA, 3.2 μ l 20% SDS and 1.5 μ l ProteinaseK (Roche Diagnostics; 20mgr/ml), samples were incubated at 50°C for 1 hr. Next, the viral DNA was isolated using the GENECLEAN spin kit (Bio101 Inc.) according to the manufacturer's instructions. DNA was eluted from the spin column with 25 μ l sterile MilliQ water. The total sequence was generated by Qiagen Sequence Services (Qiagen GmbH, Germany). Total viral DNA was sheared by sonification and the ends of the DNA were made blunt by T4 DNA polymerase. Sheared blunt fragments were size fractionated on agarose gels and gel slices corresponding to DNA fragments of 1.8 to 2.2kb were obtained. DNA was purified from the gel slices by the QIAquick gel extraction protocol

and subcloned into a shotgun library of pUC19 plasmid cloning vectors. An array of clones in 96-well plates covering the target DNA 8 (+/- 2) times was used to generate the total sequence. Sequencing was performed on Perkin-Elmer 9700 thermocyclers using Big Dye Terminator chemistry and AmpliTaq FS DNA polymerase followed by purification of sequencing reactions using QIAGEN DyeEx 96 technology. Sequencing reaction products were then subjected to automated separation and detection of fragments on ABI 377 XL 96 lane sequencers. Initial sequence results were used to generate a contiguous sequence and gaps were filled in by primer walking reads on the target DNA or by direct sequencing of PCR products. The ends of the virus turned out to be absent in the shotgun library, most probably due to cloning difficulties resulting from the amino acids of pTP that remain bound to the ITR sequences after proteinase K digestion of the viral DNA. Additional sequence runs on viral DNA solved most of the sequence in those regions, however, it was difficult to obtain a clear sequence of the most terminal nucleotides. At the 5' end the sequence portion obtained was 5'-CCAATAATATACCT-3' (SEQ. I.D. NO. __) while at the 3' end, the obtained sequence portion was 5'-AGGTATATTATTGATGATGGG-3' (SEQ. I.D. NO. __). Most human adenoviruses have a terminal sequence 5'-CATCATCAATAATATACC-3' (SEQ. I.D. NO. __). In addition, a clone representing the 3' end of the Ad35 DNA obtained after cloning the terminal 7 kb Ad35 EcoRI fragment into pBr322 also turned out to have the typical CATCATCAATAAT... sequence. Therefore, Ad35 may have the typical end sequence and the differences obtained in sequencing directly on the viral DNA are due to artifacts correlated with run-off sequence runs and the presence of residual amino acids of pTP.

[0089] The total sequence of Ad35 with corrected terminal sequences is given in FIG. 5. Based sequence homology with Ad5 (Genbank # M72360) and Ad7 (partial sequence Genbank # X03000) and on the location of open reading frames, the organization of the virus is identical to the general organization of most human adenoviruses, especially the subgroup B viruses. The total length of the genome is 34,794 basepairs.

Example 4

Construction of a plasmid-based vector system to generate recombinant Ad35-based viruses.

[0090] A functional plasmid-based vector system to generate recombinant adenoviral vectors comprises the following components:

1. An adapter plasmid comprising a left ITR and packaging sequences derived from Ad35 and at least one restriction site for insertion of a heterologous expression cassette and lacking E1 sequences. Furthermore, the adapter plasmid contains Ad35 sequences 3' from the E1B coding region including the pIX promoter and coding sequences enough to mediate homologous recombination of the adapter plasmid with a second nucleic acid molecule.
2. A second nucleic acid molecule, comprising sequences homologous to the adapter plasmid, and Ad35 sequences necessary for the replication and packaging of the recombinant virus, that is, early, intermediate and late genes that are not present in the packaging cell.
3. A packaging cell providing at least functional E1 proteins capable of complementing the E1 function of Ad35.

[0091] Other methods for generating recombinant adenoviruses on complementing packaging cells are known in the art and may be applied to Ad35 viruses without departing from the invention. As an example, the construction of a plasmid-based system, as outlined above, is described in detail below.

1) Construction of Ad35 adapter plasmids.

[0092] The adapter plasmid pAdApt (described in International Patent Application WO99/55132) was first modified to obtain adapter plasmids that contain extended polylinkers and that have convenient unique restriction sites flanking the left ITR and the adenovirus sequence at the 3' end to enable liberation of the adenovirus insert from plasmid vector sequences. Construction of these plasmids is described below in detail:

[0093] Adapter plasmid pAdApt was digested with SalI and treated with Shrimp Alkaline Phosphatase to reduce religation. A linker, composed of the following

two phosphorylated and annealed oligos: ExSalPacF 5' – TCG ATG GCA AAC AGC TAT TAT GGG TAT TAT GGG TTC GAA TTA ATT AA- 3' (SEQ. I.D. NO. __) and ExSalPacR 5' – TCG ATT AAT TAA TTC GAA CCC ATA ATA CCC ATA ATA GCT GTT TGC CA- 3' (SEQ. I.D. NO. __), was directly ligated into the digested construct, thereby replacing the SalI restriction site by Pi-PspI, SmaI and PacI. This construct was designated pADAPT+ExSalPac linker. Furthermore, part of the left ITR of pAdApt was amplified by PCR using the following primers: PCLIPMSF: 5'- CCC CAA TTG GTC GAC CAT CAT CAA TAA TAT ACC TTA TTT TGG -3' (SEQ. I.D. NO. __) and pCLIPBSRGI: 5'- GCG AAA ATT GTC ACT TCC TGT G - 3' (SEQ. I.D. NO. __). The amplified fragment was digested with MunI and BsrGI and cloned into pAd5/Clip (described in International Patent Application WO99/55132), which was partially digested with EcoRI and after purification digested with BsrGI, thereby re-inserting the left ITR and packaging signal. After restriction enzyme analysis, the construct was digested with ScaI and SgrAI and an 800 bp fragment was isolated from gel and ligated into ScaI/SgrAI digested pADAPT+ExSalPac linker. The resulting construct, designated pIPspSalAdapt, was digested with SalI, dephosphorylated, and ligated to the phosphorylated ExSalPacF/ExSalPacR double-stranded linker previously mentioned. A clone in which the PacI site was closest to the ITR was identified by restriction analysis and sequences were confirmed by sequence analysis. This novel pAdApt construct, termed pIPspAdapt, thus harbours two ExSalPac linkers containing recognition sequences for PacI, PI-PspI and BstBI, which surround the adenoviral part of the adenoviral adapter construct, and which can be used to linearize the plasmid DNA prior to co-transfection with adenoviral helper fragments.

[0094] In order to further increase transgene cloning permutations, a number of polylinker variants were constructed based on pIPspAdapt. For this purpose, pIPspAdapt was first digested with EcoRI and dephosphorylated. A linker composed of the following two phosphorylated and annealed oligos: Ecolinker+: 5' –AAT TCG GCG CGC CGT CGA CGA TAT CGA TAG CGG CCG C –3' (SEQ. I.D. NO. __) and Ecolinker-: 5' –AAT TGC GGC CGC TAT CGA TAT CGT CGA CGG CGC GCC G – 3' (SEQ. I.D. NO. __) was ligated into this construct, thereby creating restriction sites for AscI, SalI, EcoRV, ClaI and NotI. Both orientations of this linker were obtained, and

sequences were confirmed by restriction analysis and sequence analysis. The plasmid containing the polylinker in the order 5' HindIII, KpnI, AgeI, EcoRI, AscI, SalI, EcoRV, ClaI, NotI, NheI, HpaI, BamHI and XbaI was termed pIPspAdapt1, while the plasmid containing the polylinker in the order HindIII, KpnI, AgeI, NotI, ClaI, EcoRV, SalI, AscI, EcoRI, NheI, HpaI, BamHI and XbaI was termed pIPspAdapt2.

[0095] To facilitate the cloning of other sense or antisense constructs, a linker composed of the following two oligonucleotides was designed to reverse the polylinker of pIPspAdapt: HindXba+ 5'-AGC TCT AGA GGA TCC GTT AAC GCT AGC GAA TTC ACC GGT ACC AAG CTT A-3' (SEQ. I.D. NO. __); HindXba- 5'-CTA GTA AGC TTG GTA CCG GTG AAT TCG CTA GCG TTA ACG GAT CCT CTA G-3' (SEQ. I.D. NO. __). This linker was ligated into HindIII/XbaI digested pIPspAdapt and the correct construct was isolated. Confirmation was done by restriction enzyme analysis and sequencing. This new construct, pIPspAdaptA, was digested with EcoRI and the previously mentioned Ecolinker was ligated into this construct. Both orientations of this linker were obtained, resulting in pIPspAdapt3, which contains the polylinker in the order XbaI, BamHI, HpaI, NheI, EcoRI, AscI, SalI, EcoRV, ClaI, NotI, AgeI, KpnI and HindIII. All sequences were confirmed by restriction enzyme analysis and sequencing.

[0096] Adapter plasmids based on Ad35 were then constructed as follows:

[0097] The left ITR and packaging sequence corresponding to Ad35 wt sequences nucleotides 1 to 464 (FIG. 5) were amplified by PCR on wt Ad35 DNA using the following primers:

Primer 35F1:

5'-CGG AAT TCT TAA TTA ATC GAC ATC ATC AAT AAT ATA CCT TAT AG-3' (SEQ. I.D. NO. __)

Primer 35R2:

5'-GGT GGT CCT AGG CTG ACA CCT ACG TAA AAA CAG-3' (SEQ. I.D. NO. __)

Amplification introduces a PacI site at the 5' end and an AvrII site at the 3' end of the sequence.

[0098] For the amplification, Platinum Pfx DNA polymerase enzyme (LTI) was used according to manufacturer's instructions, but with primers at 0.6 μ M and with DMSO added to a final concentration of 3%. Amplification program was as follows: 2

min. at 94°C, (30 sec. 94°C, 30 sec. at 56°C, 1 min. at 68°C) for 30 cycles, followed by 10 min. at 68°C.

[0099] The PCR product was purified using a PCR purification kit (LTI) according to the manufacturer's instructions and digested with *PacI* and *AvrII*. The digested fragment was then purified from gel using the GENECLEAN kit (Bio 101, Inc.). The Ad5-based adapter plasmid pIPspAdApt-3 was digested with *AvrII* and then partially with *PacI* and the 5762 bp fragment was isolated in an LMP agarose gel slice and ligated with the above-mentioned PCR fragment digested with the same enzymes and transformed into electrocompetent DH10B cells (LTI). The resulting clone is designated pIPspAdApt3-Ad35ITR.

[0100] In parallel, a second piece of Ad35 DNA was amplified using the following primers:

35F3: 5'- TGG TGG AGA TCT GGT GAG TAT TGG GAA AAC-3' (SEQ. I.D. NO.)

35R4: 5'- CGG AAT TCT TAA TTA AGG GAA ATG CAA ATC TGT GAG G-3' (SEQ. I.D. NO.)

[0101] The sequence of this fragment corresponds to nucleotides 3401 to 4669 of wt Ad35 (FIG. 5) and contains 1.3kb of sequences starting directly 3' from the E1B-55k coding sequence. Amplification and purification were done as previously described herein for the fragment containing the left ITR and packaging sequence. The PCR fragment was then digested with *PacI* and subcloned into pNEB193 vector (New England Biolabs) digested with *SmaI* and *PacI*. The integrity of the sequence of the resulting clone was checked by sequence analysis. pNEB/Ad35pF3R4 was then digested with *BglII* and *PacI* and the Ad35 insert was isolated from gel using the QIAExII kit (Qiagen). pIPspAdApt3-Ad35ITR was digested with *BglII* and then partially with *PacI*. The 3624 bp fragment (containing vector sequences, the Ad35 ITR and packaging sequences as well as the CMV promoter, multiple cloning region and polyA signal) was also isolated using the QIAExII kit (Qiagen). Both fragments were ligated and transformed into competent DH10B cells (LTI). The resulting clone, pAdApt35IP3, has the expression cassette from pIPspAdApt3 but contains the Ad35 left ITR and packaging sequences and a second fragment corresponding to nucleotides 3401 to 4669 from Ad35.

A second version of the Ad35 adapter plasmid having the multiple cloning site in the opposite orientation was made as follows:

[0102] pIPspAdapt1 was digested with NdeI and BglII and the 0.7 kbp band containing part of the CMV promoter, the MCS and SV40 polyA was isolated and inserted in the corresponding sites of pAdApt35IP3 generating pAdApt35IP1 (Fig. 6).

[0103] pAdApt35.LacZ and pAdApt35.Luc adapter plasmids were then generated by inserting the transgenes from pcDNA.LacZ (digested with KpnI and BamHI) and pAdApt.Luc (digested with HindIII and BamHI) into the corresponding sites in pAdApt35IP1. The generation of pcDNA.LacZ and pAdApt.Luc is described in International Patent Application WO99/55132.

2) Construction of cosmid pWE.Ad35.pIX-rITR

[0104] FIG. 7 presents the various steps undertaken to construct the cosmid clone containing Ad35 sequences from bp 3401 to 34794 (end of the right ITR) that are described in detail below.

[0105] A first PCR fragment (pIX-NdeI) was generated using the following primer set:

35F5: 5'-CGG AAT TCG CGG CCG CGG TGA GTA TTG GGA AAA C -3' (SEQ. I.D. NO. __)

35R6: 5'-CGC CAG ATC GTC TAC AGA ACA G-3' (SEQ. I.D. NO. __)

[0106] DNA polymerase Pwo (Roche) was used according to manufacturer's instructions, however, with an end concentration of 0.6 μ M of both primers and using 50 ngr wt Ad35 DNA as template. Amplification was done as follows: 2 min. at 94 °C, 30 cycles of 30 sec. at 94 °C, 30 sec. at 65 °C and 1 min. 45 sec. at 72 °C, followed by 8 min. at 68 °C. To enable cloning in the TA cloning vector PCR2.1, a last incubation with 1 unit superTaq polymerase (HT Biotechnology LTD) for 10 min. at 72 °C was performed.

[0107] The 3370 bp amplified fragment contains Ad35 sequences from bp 3401 to 6772 with a NotI site added to the 5' end. Fragments were purified using the PCR purification kit (LTI).

[0108] A second PCR fragment (NdeI-rITR) was generated using the following primers:

35F7: 5'-GAA TGC TGG CTT CAG TTG TAA TC -3' (SEQ. I.D. NO. __)

35R8: 5'- CGG AAT TCG CGG CCG CAT TTA AAT CAT CAT CAA TAA TAT ACC-3' (SEQ. I.D. NO. __)

[0109] Amplification was done with pfx DNA polymerase (LTI) according to manufacturer's instructions but with 0.6 μ M of both primers and 3% DMSO using 10 ngr. of wt Ad35 DNA as template. The program was as follows: 3 min. at 94 °C and 5 cycles of 30 sec. at 94 °C, 45 sec. at 40 °C, 2 min.45 sec. at 68 °C followed by 25 cycles of 30 sec. at 94 °C, 30 sec. at 60 °C, 2 min.45 sec. at 68 °C. To enable cloning in the TA-cloning vector PCR2.1, a last incubation with 1 unit superTaq polymerase for 10 min. at 72 °C was performed. The 1.6 kb amplified fragment ranging from nucleotides 33178 to the end of the right ITR of Ad35, was purified using the PCR purification kit (LTI).

[0110] Both purified PCR fragments were ligated into the PCR2.1 vector of the TA-cloning kit (Invitrogen) and transformed into STBL-2 competent cells (LTI). Clones containing the expected insert were sequenced to confirm correct amplification. Next, both fragments were excised from the vector by digestion with NotI and NdeI and purified from gel using the GENECLAN kit (BIO 101, Inc.). Cosmid vector pWE15 (Clontech) was digested with NotI, dephosphorylated and also purified from gel. These three fragments were ligated and transformed into STBL2 competent cells (LTI). One of the correct clones that contained both PCR fragments was then digested with NdeI, and the linear fragment was purified from gel using the GENECLAN kit. Ad35 wt DNA was digested with NdeI and the 26.6 kb fragment was purified from LMP gel using agarase enzyme (Roche) according to the manufacturer's instructions. These fragments were ligated together and packaged using λ 1 phage packaging extracts (Stratagene) according to the manufacturer's protocol. After infection into STBL-2 cells, colonies were grown on plates and analysed for presence of the complete insert. One clone with the large fragment inserted in the correct orientation and having the correct restriction patterns after independent digestions with three enzymes (NcoI, PvuII and ScaI) was selected. This clone is designated pWE.Ad35.pIX-rITR. It contains the Ad35 sequences from bp 3401 to the end and is flanked by NotI sites (FIG. 8).

3) Generation of Ad35-based recombinant viruses on PER.C6.

[0111] Wild type Ad35 virus can be grown on PER.C6 packaging cells to very high titers. However, whether the Ad5-E1 region that is present in PER.C6 is able to complement E1-deleted Ad35 recombinant viruses is unknown. To test this, PER.C6 cells were cotransfected with the above-described adapter plasmid pAdApt35.LacZ and the large backbone fragment pWE.Ad35.pIX-rITR. First, pAdApt35.LacZ was digested with PacI and pWE.Ad35.pIX-rITR was digested with NotI. Without further purification, 4 µgr of each construct was mixed with DMEM (LTI) and transfected into PER.C6 cells, seeded at a density of 5×10^6 cells in a T25 flask the day before, using Lipofectamin (LTI) according to the manufacturer's instructions. As a positive control, 6µgr of PacI digested pWE.Ad35.pIX-rITR DNA was cotransfected with a 6.7 kb NheI fragment isolated from Ad35 wt DNA containing the left end of the viral genome including the E1 region. The next day, medium (DMEM with 10% FBS and 10mM MgCl₂) was refreshed and cells were further incubated. At day 2 following the transfection, cells were trypsinized and transferred to T80 flasks. The positive control flask showed CPE at five days following transfection, showing that the pWE.Ad35.pIX-rITR construct is functional, at least in the presence of Ad35-E1 proteins. The transfection with the Ad35 LacZ adapter plasmid and pWE.Ad35.pIX-rITR did not give rise to CPE. These cells were harvested in the medium at day 10 and freeze/thawed once to release virus from the cells. 4 ml of the harvested material was added to a T80 flask with PER.C6 cells (at 80% confluency) and incubated for another five days. This harvest/re-infection was repeated two times but there was no evidence for virus associated CPE.

[0112] From this experiment, it seems that the Ad5-E1 proteins are not, or not well enough, capable of complementing Ad35 recombinant viruses. However, it may be that the sequence overlap of the adapter plasmid and the pWE.Ad35.pIX-rITR backbone plasmid is not large enough to efficiently recombine and give rise to a recombinant virus genome. The positive control transfection was done with a 6.7kb left end fragment and, therefore, the sequence overlap was about 3.5kb. The adapter plasmid and the pWE.Ad35.pIX-rITR fragment have a sequence overlap of 1.3kb. To check whether the sequence overlap of 1.3 kb is too small for efficient homologous recombination, a co-transfection was done with PacI digested pWE.Ad35.pIX-rITR and a PCR fragment of

Ad35 wt DNA generated with the above-mentioned 35F1 and 35R4 using the same procedures as previously described herein. The PCR fragment thus contains left end sequences up to bp 4669 and, therefore, has the same overlap sequences with pWE.Ad35.pIX-rITR as the adapter plasmid pAdApt35.LacZ, but has Ad35-E1 sequences. Following PCR column purification, the DNA was digested with SalI to remove possible intact template sequences. A transfection with the digested PCR product alone served as a negative control. Four days after the transfection, CPE occurred in the cells transfected with the PCR product and the Ad35 pIX-rITR fragment, and not in the negative control. This result shows that a 1.3kb overlapping sequence is sufficient to generate viruses in the presence of Ad35-E1 proteins. From these experiments, we conclude that the presence of at least one of the Ad35-E1 proteins is necessary to generate recombinant Ad35 based vectors from plasmid DNA on Ad5 complementing cell lines.

Example 5

1) Construction of Ad35-E1 expression plasmids

[0113] Since Ad5-E1 proteins in PER.C6 are incapable of complementing Ad35 recombinant viruses efficiently, Ad35-E1 proteins have to be expressed in Ad5 complementing cells (*e.g.*, PER.C6). Alternatively, a new packaging cell line expressing Ad35-E1 proteins has to be made, starting from either diploid primary human cells or established cell lines not expressing adenovirus E1 proteins. To address the first possibility, the Ad35-E1 region was cloned in expression plasmids as described below.

[0114] First, the Ad35-E1 region from bp 468 to bp 3400 was amplified from wt Ad35 DNA using the following primer set:

35F11: 5'-GGG GTA CCG AAT TCT CGC TAG GGT ATT TAT ACC-3' (SEQ. I.D. NO. __)

35F10: 5'-GCT CTA GAC CTG CAG GTT AGT CAG TTT CTT CTC CAC TG-3' (SEQ. I.D. NO. __)

[0115] This PCR introduces a KpnI and EcoRI site at the 5' end and an SbfI and XbaI site at the 3' end.

[0116] Amplification on 5 ngr. template DNA was done with Pwo DNA polymerase (Roche) using the manufacturer's instructions, however, with both primers at a final concentration of 0.6 μ M. The program was as follows: 2 min. at 94 °C, 5 cycles of 30 sec. at 94 °C, 30 sec. at 56 °C and 2 min. at 72 °C, followed by 25 cycles of 30 sec. at 94°C, 30 sec. at 60 °C and 2 min. at 72 °C, followed by 10 min. at 72 °C. PCR product was purified by a PCR purification kit (LTI) and digested with KpnI and XbaI. The digested PCR fragment was then ligated to the expression vector pRSVhbvNeo (see below) also digested with KpnI and XbaI. Ligations were transformed into competent STBL-2 cells (LTI) according to manufacturer's instructions and colonies were analysed for the correct insertion of Ad35-E1 sequences into the polylinker in between the RSV promoter and HBV polyA.

[0117] The resulting clone was designated pRSV.Ad35-E1 (FIG. 9). The Ad35 sequences in pRSV.Ad35-E1 were checked by sequence analysis.

[0118] pRSVhbvNeo was generated as follows: pRc-RSV (Invitrogen) was digested with PvuII, dephosphorylated with TSAP enzyme (LTI), and the 3kb vector fragment was isolated in low melting point agarose (LMP). Plasmid pPGKneopA (FIG. 10; described in International Patent Application WO96/35798) was digested with SspI completely to linearize the plasmid and facilitate partial digestion with PvuII. Following the partial digestion with PvuII, the resulting fragments were separated on a LMP agarose gel and the 2245 bp PvuII fragment, containing the PGK promoter, neomycin-resistance gene and HBVpolyA, was isolated. Both isolated fragments were ligated to give the expression vector pRSV-pNeo that now has the original SV40prom-neo-SV40polyA expression cassette replaced by a PGKprom-neo-HBVpolyA cassette (FIG. 11). This plasmid was further modified to replace the BGHpA with the HBVpA as follows: pRSVpNeo was linearised with ScaI and further digested with XbaI. The 1145 bp fragment, containing part of the Amp gene and the RSV promoter sequences and polylinker sequence, was isolated from gel using the GENE CLEAN kit (Bio Inc. 101). Next, pRSVpNeo was linearised with ScaI and further digested partially with EcoRI and the 3704 bp fragment containing the PGKneo cassette and the vector sequences were isolated from gel as above. A third fragment, containing the HBV polyA sequence

flanked by XbaI and EcoRI at the 5' and 3' end, respectively, was then generated by PCR amplification on pRSVpNeo using the following primer set:

HBV-F: 5'- GGC TCT AGA GAT CCT TCG CGG GAC GTC -3' (SEQ. I.D. NO. __)

and

HBV-R: 5'- GGC GAA TTC ACT GCC TTC CAC CAA GC -3' (SEQ. I.D. NO. __).

[0119] Amplification was done with Elongase enzyme (LTI) according to the manufacturer's instructions with the following conditions: 30 seconds at 94°C, then 5 cycles of 45 seconds at 94 °C, 1 minute at 42 °C and 1 minute at 68 °C, followed by 30 cycles of 45 seconds at 94 °C, 1 minute at 65 °C and 1 minute at 68 °C, followed by 10 minutes at 68 °C. The 625 bp PCR fragment was then purified using the Qiaquick PCR purification kit, digested with EcoRI and XbaI and purified from gel using the GENECLAN kit. The three isolated fragments were ligated and transformed into DH5α competent cells (LTI) to give the construct pRSVhbvNeo (FIG. 12). In this construct, the transcription regulatory regions of the RSV expression cassette and the neomycin selection marker are modified to reduce overlap with adenoviral vectors that often contain CMV and SV40 transcription regulatory sequences.

2) Generation of Ad35 recombinant viruses on PER.C6 cells cotransfected with an Ad35-E1 expression construct.

[0120] PER.C6 cells were seeded at a density of 5×10^6 cells in a T25 flask and, the next day, transfected with a DNA mixture containing:

1 µg pAdApt35.LacZ digested with PacI

5 µg pRSV.Ad35E1 undigested

2 µg pWE.Ad35.pIX-rITR digested with NotI

[0121] Transfection was done using Lipofectamine according to the manufacturer's instructions. Five hours after addition of the transfection mixture to the cells, medium was removed and replaced by fresh medium. After two days, cells were transferred to T80 flasks and further cultured. One week post-transfection, 1 ml of the medium was added to A549 cells and, the following day, cells were stained for LacZ expression. Blue cells were clearly visible after two hours of staining indicating that recombinant LacZ expressing viruses were produced. The cells were further cultured, but

no clear appearance of CPE was noted. However, after 12 days, clumps of cells appeared in the monolayer and 18 days following transfection, cells were detached. Cells and medium were then harvested, freeze-thawed once, and 1 ml of the crude lysate was used to infect PER.C6 cells in a 6-well plate. Two days after infection, cells were stained for LacZ activity. After two hours, 15% of the cells were stained blue. To test for the presence of wt and / or replicating competent viruses, A549 cells were infected with these viruses and further cultured. No signs of CPE were found indicating the absence of replication-competent viruses. These experiments show that recombinant AdApt35.LacZ viruses were made on PER.C6 cells cotransfected with an Ad35-E1 expression construct.

[0122] Ad35 recombinant viruses escape neutralization in human serum containing neutralizing activity to Ad5 viruses.

[0123] The AdApt35.LacZ viruses were then used to investigate infection in the presence of serum that contains neutralizing activity to Ad5 viruses. Purified Ad5-based LacZ virus served as a positive control for NA. Hereto, PER.C6 cells were seeded in a 24-well plate at a density of 2×10^5 cells/well. The next day, a human serum sample with high neutralizing activity to Ad5 was diluted in culture medium in five steps of five times dilutions. 0.5 ml of diluted serum was then mixed with 4×10^6 virus particles AdApt5.LacZ virus in 0.5 ml medium and after 30 minutes of incubation at 37 °C, 0.5 ml of the mixture was added to PER.C6 cells in duplicate. For the AdApt35.LacZ viruses, 0.5 ml of the diluted serum samples were mixed with 0.5 ml crude lysate containing AdApt35.LacZ virus and, after incubation, 0.5 ml of this mixture was added to PER.C6 cells *in duplo*. Virus samples incubated in medium without serum were used as positive controls for infection. After two hours of infection at 37 °C, medium was added to reach a final volume of 1 ml and cells were further incubated. Two days after infection, cells were stained for LacZ activity. The results are shown in Table II. From these results, it is clear that whereas AdApt5.LacZ viruses are efficiently neutralized, AdApt35.LacZ viruses remain infectious irrespective of the presence of human serum. This proves that recombinant Ad35-based viruses escape neutralization in human sera that contain NA to Ad5-based viruses.

Example 6

Generation of cell lines capable of complementing E1-deleted Ad35 viruses

Generation of pIG135 and pIG270

[0124] Construct pIG.E1A.E1B (FIG. 13) contains E1 region sequences of Ad5 corresponding to nucleotides 459 to 3510 of the wt Ad5 sequence (Genbank accession number M72360) operatively linked to the human phosphoglycerate kinase promoter ("PGK") and the Hepatitis B Virus polyA sequences. The generation of this construct is described in International Patent Application No. WO97/00326. The E1 sequences of Ad5 were replaced by corresponding sequences of Ad35 as follows. pRSV.Ad35-E1 (described in Example 5) was digested with EcoRI and Sse8387I and the 3 kb fragment corresponding to the Ad35-E1 sequences was isolated from gel. Construct pIG.E1A.E1B was digested with Sse8387I completely and partially with EcoRI. The 4.2 kb fragment corresponding to vector sequences without the Ad5-E1 region but retaining the PGK promoter were separated from other fragments on LMP agarose gel and the correct band was excised from gel. Both obtained fragments were ligated resulting in pIG.Ad35-E1.

[0125] This vector was further modified to remove the LacZ sequences present in the pUC119 vector backbone. Hereto, the vector was digested with BsaAI and BstXI and the large fragment was isolated from gel. A double stranded oligo was prepared by annealing the following two oligos:

BB1: 5'-GTG CCT AGG CCA CGG GG-3' (SEQ. I.D. NO. __) and

BB2: 5'-GTG GCC TAG GCA C-3' (SEQ. I.D. NO. __).

[0126] Ligation of the oligo and the vector fragment resulted in construct pIG135 (FIG. 14). Correct insertion of the oligo restores the BsaAI and BstXI sites and introduces a unique AvrII site. Next, we introduced a unique site at the 3' end of the Ad35-E1 expression cassette in pIG135. Hereto, the construct was digested with SapI and the 3' protruding ends were made blunt by treatment with T4 DNA polymerase. The thus treated linear plasmid was further digested with BsrGI and the large vector-containing fragment was isolated from gel. To restore the 3' end of the HBVpolyA sequence and to introduce a unique site, a PCR fragment was generated using the following primers:

270F: 5'-CAC CTC TGC CTA ATC ATC TC -3' (SEQ. I.D. NO. __) and

270R: 5'- GCT CTA GAA ATT CCA CTG CCT TCC ACC -3' (SEQ. I.D. NO. ____).

[0127] The PCR was performed on pIG.Ad35.E1 DNA using Pwo polymerase (Roche) according to the manufacturer's instructions. The obtained PCR product was digested with BsrGI and dephosphorylated using Tsap enzyme (LTI), the latter to prevent insert dimerization on the BsrGI site. The PCR fragment and the vector fragment were ligated to yield construct pIG270 (FIG. 15).

Ad35-E1 sequences are capable of transforming rat primary cells

[0128] Newborn WAG/RIJ rats were sacrificed at 1 week of gestation and kidneys were isolated. After careful removal of the capsule, kidneys were disintegrated into a single cell suspension by multiple rounds of incubation in trypsin/EDTA (LTI) at 37 °C and collection of floating cells in cold PBS containing 1% FBS. When most of the kidney was trypsinized, all cells were re-suspended in DMEM supplemented with 10% FBS and filtered through a sterile cheesecloth. Baby Rat Kidney (BRK) cells obtained from one kidney were plated in 5 dishes (Greiner, 6 cm). When a confluency of 70-80% was reached, the cells were transfected with 1 or 5 µgr DNA/dish using the CaPO₄ precipitation kit (LTI) according to the manufacturer's instructions. The following constructs were used in separate transfections: pIG.E1A.E1B (expressing the Ad5-E1 region), pRSV.Ad35-E1, pIG.Ad35-E1 and pIG270 (expressing the Ad35-E1 region). Cells were incubated at 37 °C, 5% CO₂ until foci of transformed cells appeared. Table III shows the number of foci that resulted from several transfection experiments using circular or linear DNA. As expected, the Ad5-E1 region efficiently transformed BRK cells. Foci also appeared in the Ad35-E1 transfected cell layer although with lower efficiency. The Ad35 transformed foci appeared at a later time point: ~2 weeks post transfection compared with 7-10 days for Ad5-E1. These experiments clearly show that the E1 genes of the B group virus Ad35 are capable of transforming primary rodent cells. This proves the functionality of the Ad35-E1 expression constructs and confirms earlier findings of the transforming capacity of the B-group viruses Ad3 and Ad7 (Dijkema, 1979). To test whether the cells in the foci were really transformed, a few foci were picked and expanded. From the 7 picked foci, at least 5 turned out to grow as established cell lines.

Generation of new packaging cells derived from primary human amniocytes

[0129] Amniotic fluid obtained after amniocentesis was centrifuged and cells were re-suspended in AmnioMax medium (LTI) and cultured in tissue culture flasks at 37 °C and 10 % CO₂. When cells were growing nicely (approximately one cell division/24 hrs.), the medium was replaced with a 1:1 mixture of AmnioMax complete medium and DMEM low glucose medium (LTI) supplemented with Glutamax I (end concentration 4mM, LTI) and glucose (end concentration 4.5 gr/L, LTI) and 10% FBS (LTI). For transfection ~ 5x10⁵ cells were plated in 10 cm tissue culture dishes. The day after, cells were transfected with 20 µgr of circular pIG270/dish using the CaPO₄ transfection kit (LTI) according to manufacturer's instructions and cells were incubated overnight with the DNA precipitate. The following day, cells were washed 4 times with PBS to remove the precipitate and further incubated for over three weeks until foci of transformed cells appeared. Once a week the medium was replaced by fresh medium. Other transfection agents like, but not limited to, LipofectAmine (LTI) or PEI (Polyethylenimine, high molecular weight, water-free, Aldrich) were used. Of these three agents, PEI reached the best transfection efficiency on primary human amniocytes: ~1% blue cells 48 hrs.

Following transfection of pAdApt35. LacZ.

[0130] Foci are isolated as follows. The medium is removed and replaced by PBS after which foci are isolated by gently scraping the cells using a 50-200 µl Gilson pipette with a disposable filter tip. Cells contained in ~10 µmL PBS were brought in a 96 well plate containing 15 µl trypsin/EDTA (LTI) and a single cell suspension was obtained by pipetting up and down and a short incubation at room temperature. After addition of 200 µl of the above described 1:1 mixture of AmnioMax complete medium and DMEM with supplements and 10% FBS, cells were further incubated. Clones that continued to grow were expanded and analysed for their ability to complement growth of E1-deleted adenoviral vectors of different sub-groups, specifically ones derived from B-group viruses, and more specifically from Ad35 or Ad11.

Generation of new packaging cell lines from HER cells

[0131] HER cells are isolated and cultured in DMEM medium supplemented with 10% FBS (LTI). The day before transfection, $\sim 5 \times 10^5$ cells are plated in 6 cm dishes and cultured overnight at 37 °C and 10% CO₂. Transfection is done using the CaPO₄ precipitation kit (LTI) according to the manufacturer's instructions. Each dish is transfected with 8-10 µmgr pIG270 DNA, either as a circular plasmid or as a purified fragment. To obtain the purified fragment, pIG270 was digested with AvrII and XbaI and the 4 kb fragment corresponding to the Ad35-E1 expression cassette was isolated from gel by agarase treatment (Roche). The following day, the precipitate is washed away carefully by four washes with sterile PBS. Then fresh medium is added and transfected cells are further cultured until foci of transformed cells appear. When large enough (>100 cells), foci are picked and brought into 96-wells as described above. Clones of transformed HER cells that continue to grow, are expanded and tested for their ability to complement growth of E1-deleted adenoviral vectors of different sub-groups, specifically ones derived from B-group viruses, and more specifically from Ad35 or Ad11.

New packaging cell lines derived from PER.C6

[0132] As described in Example 5, it is possible to generate and grow Ad35-E1-deleted viruses on PER.C6 cells with cotransfection of an Ad35-E1 expression construct, e.g., pRSV.Ad35.E1. However, large-scale production of recombinant adenoviruses using this method is cumbersome because, for each amplification step, a transfection of the Ad35-E1 construct is needed. In addition, this method increases the risk of non-homologous recombination between the plasmid and the virus genome with high chances of generation of recombinant viruses that incorporate E1 sequences resulting in replication-competent viruses. To avoid this, the expression of Ad35-E1 proteins in PER.C6 has to be mediated by integrated copies of the expression plasmid in the genome. Since PER.C6 cells are already transformed and express Ad5-E1 proteins, addition of extra Ad35-E1 expression may be toxic for the cells. However, it is not impossible to stably transfect transformed cells with E1 proteins since Ad5-E1-expressing A549 cells have been generated.

[0133] In an attempt to generate recombinant adenoviruses derived from subgroup B virus Ad7, Abrahamsen *et al.* (1997) were not able to generate E1-deleted viruses on 293 cells without contamination of wt Ad7. Viruses that were picked after plaque purification on 293-ORF6 cells (Brough *et al.*, 1996) were shown to have incorporated Ad7-E1B sequences by nonhomologous recombination. Thus, efficient propagation of Ad7 recombinant viruses proved possible only in the presence of Ad7-E1B expression and Ad5-E4-ORF6 expression. The E1B proteins are known to interact with cellular as well as viral proteins (Bridge *et al.*, 1993; White, 1995). Possibly, the complex formed between the E1B-55K protein and E4-ORF6 which is necessary to increase mRNA export of viral proteins and to inhibit export of most cellular mRNAs is critical and, in some way, serotype-specific. The above experiments suggest that the E1A proteins of Ad5 are capable of complementing an Ad7-E1A deletion and that Ad7-E1B expression in adenovirus packaging cells on itself is not enough to generate a stable complementing cell line. To test whether one or both of the Ad35-E1B proteins is/are the limiting factor in efficient Ad35 vector propagation on PER.C6 cells, we have generated an Ad35 adapter plasmid that does contain the E1B promoter and E1B sequences but lacks the promoter and the coding region for E1A. Hereto, the left end of wt Ad35 DNA was amplified using the primers 35F1 and 35R4 (both described in Example 4) with Pwo DNA polymerase (Roche) according to the manufacturer's instructions. The 4.6 kb PCR product was purified using the PCR purification kit (LTI) and digested with SnaBI and ApaI enzymes. The resulting 4.2 kb fragment was then purified from gel using the QIAExII kit (Qiagen). Next, pAdApt35IP1 (Example 4) was digested with SnaBI and ApaI and the 2.6 kb vector-containing fragment was isolated from gel using the GENECLEAN kit (BIO 101, Inc). Both isolated fragments were ligated to give pBr/Ad35.leftITR-pIX (FIG.16). Correct amplification during PCR was verified by a functionality test as follows: The DNA was digested with BstBI to liberate the Ad35 insert from vector sequences and 4 µg of this DNA was cotransfected with 4 µg of NotI digested pWE/Ad35.pIX-rITR (Example 4) into PER.C6 cells. The transfected cells were passaged to T80 flasks at day 2 and again two days later CPE had formed showing that the new pBr/Ad35.leftITR-pIX construct contains functional E1 sequences. The pBr/Ad35.leftITR-pIX construct was then further modified as follows. The DNA was

digested with SnaBI and HindIII and the 5' HindII overhang was filled in using Klenow enzyme. Religation of the digested DNA and transformation into competent cells (LTI) gave construct pBr/Ad35leftITR-pIXΔE1A (FIG. 17). This latter construct contains the left end 4.6 kb of Ad35 except for E1A sequences between bp 450 and 1341 (numbering according to wt Ad35, FIG. 5) and thus lacks the E1A promoter and most of the E1A coding sequences. pBr/Ad35.leftITR-pIXΔE1A was then digested with BstBI and 2 μg of this construct was cotransfected with 6 μg of NotI digested pWE/Ad35.pIX-rITR (Example 4) into PER.C6 cells. One week following transfection, full CPE had formed in the transfected flasks.

[0134] This experiment shows that the Ad35-E1A proteins are functionally complemented by Ad5-E1A expression in PER.C6 cells and that at least one of the Ad35-E1B proteins cannot be complemented by Ad5-E1 expression in PER.C6. It further shows that it is possible to make a complementing cell line for Ad35-E1-deleted viruses by expressing Ad35-E1B proteins in PER.C6. Stable expression of Ad35-E1B sequences from integrated copies in the genome of PER.C6 cells may be driven by the E1B promoter and terminated by a heterologous poly-adenylation signal like, but not limited to, the HBVpA. The heterologous pA signal is necessary to avoid overlap between the E1B insert and the recombinant vector, since the natural E1B termination is located in the pIX transcription unit that has to be present on the adenoviral vector. Alternatively, the E1B sequences may be driven by a heterologous promoter like, but not limited to, the human PGK promoter or by an inducible promoter like, but not limited to, the 7xtetO promoter (Gossen and Bujard, 1992). Also, in these cases, the transcription termination is mediated by a heterologous pA sequence, *e.g.*, the HBV pA. The Ad35-E1B sequences at least comprise one of the coding regions of the E1B-21K and the E1B-55K proteins located between nucleotides 1611 and 3400 of the wt Ad35 sequence. The insert may also include part of the Ad35-E1B sequences between nucleotides 1550 and 1611 of the wt Ad35 sequence.

Example 7

Ad35-based viruses deleted for E1A and E1B-21K genes efficiently propagate on Ad5 complementing cell lines.

[0135] The generation of Ad35-based viruses that are deleted for E1A and retain the full E1B region is described in Example 6 of this application. Such viruses can be generated and propagated on the Ad5 complementing cell line PER.C6. The E1B region comprises partially overlapping coding sequences for the two major proteins 21K and 55K (Bos *et al.*, 1981). Whereas, during productive wt adenoviral infection, both 21K and 55K are involved in counteracting the apoptose-inducing effects of E1A proteins, the E1B-55K protein has been suggested to have additional functions during the late phase of virus infection. These include the accumulation of viral mRNAs, the control of late viral gene expression and the shutoff of most host mRNAs at the level of mRNA transport (Babiss *et al.*, 1984, 1985; Pilder *et al.*, 1986). A complex formed between E1B-55K and the ORF6 protein encoded by the adenovirus early region 4 (Leppard and Shenk, 1989; Bridge and Ketner, 1990) exerts at least part of these functions.

[0136] To analyze which of the E1B proteins is required for propagation of Ad35-E1A-deleted recombinant viruses on PER.C6 packaging cells, the E1B region in construct pBr.Ad35.leftITR-pIXΔE1A (see Example 6 and FIG. 17) was further deleted. A first construct, pBr.Ad35Δ21K, retains the full E1B-55K sequence and is deleted for E1A and E1B-21K. Hereto, pBr.Ad35.leftITR-pIXΔE1A was digested with NcoI and BspE1 and the 5 KB vector fragment was isolated from agarose gel using the GENECLEAN kit (BIO 101, Inc.) according to the manufacturer's instructions. Then a PCR fragment was generated with pBr.Ad35.leftITR-pIXΔE1A as template DNA using the following primers:

35D21: 5'- TTA GAT CCA TGG ATC CCG CAG ACT C-3' (SEQ. I.D. NO. __) and
35B3: 5'- CCT CAG CCC CAT TTC CAG-3' (SEQ. I.D. NO. __).

Amplification was done using Pwo DNA polymerase (Roche) according to manufacturer's recommendations with the addition of DMSO (final concentration 3%) in the reaction mixture. The PCR program was as follows: 94°C for 2', then 30 cycles of 94°C for 30'', 58°C for 30'' and 72°C for 45'' and a final step at 68°C for 8' to ensure blunt ends.

[0137] This PCR amplifies Ad35-E1B sequences from nucl. 1908 to 2528 (sequence Ad35, FIG. 5) and introduces an NcoI site at the start codon of the E1B-55K coding sequence (bold in primer 35D21). The 620 bp PCR fragment was purified using the PCR purification kit (Qiagen) and then digested with NcoI and BspEI, purified from agarose gel as above and ligated to the above-described NcoI/BspEI digested vector fragment to give pBr.Ad35Δ21K (FIG. 18).

[0138] Since the coding regions of the 21K and 55K proteins overlap, it is only possible to delete part of the 55K coding sequences while retaining 21K. Hereto, pBr.Ad35.leftITR-pIXΔE1A was digested with BglII and the vector fragment was religated to give pBr.Ad35Δ55K1 (FIG. 19). This deletion removes E1B coding sequences from nucl. 2261 to 3330 (Ad35 sequence in FIG. 5). In this construct the N-terminal 115 amino acids are retained and become fused to 21 additional amino acids out of the proper reading frame before a stop codon is encountered. The 21K coding region is intact in construct pBr.Ad35Δ55K1.

[0139] A third construct that has a deletion of E1A, 21K and most of the 55K sequences was generated as follows. pBr.Ad35.leftITR-pIX (FIG. 16) was digested with SnaBI and MfeI (isoschizomer of MunI) and the 5' overhang resulting from the MfeI digestion was filled in using Klenow enzyme. The 4.4 kb vector fragment was isolated from gel using the GENE CLEAN kit (Bio 101, Inc.) according to the manufacturer's instructions and religated to give construct pBr.Ad35ΔSM (FIG. 20). In this construct, the Ad35 sequences between nucl. 453 and 2804 are deleted. Thus, 596 nucl. of the 3' end of E1b-55K are retained. A further deletion of 55K sequences was made in construct pBr.Ad35ΔE1A.ΔE1B by digestion of pBr.Ad35.leftITR-pIX with SnaBI and BglII, Klenow treatment to fill in the BglII cohesive ends, and religation. FIG. 21 shows a schematic representation of the above-mentioned constructs.

[0140] To test whether Ad35-based viruses can be generated with these constructs, each of the constructs was cotransfected with NotI digested pWE.Ad35pIX-rITR (see Example 4) onto PER.C6 cells. Hereto, the respective fragments were PCR amplified using primers 35F1 and 35R4 (see, Example 4). This PCR amplification was done since some of the constructs were difficult to isolate in large enough quantities. In

this way, equal quality of the different adapter fragments was ensured. For the amplification, Pwo DNA polymerase (Roche) was used according to the manufacturer's instructions but with DMSO (3% final concentration) added to the PCR mixture. Of each template ~ 50 ng DNA was used. The conditions for the PCR were as follows: 94°C for 2', then 5 cycles of 94°C for 30'', 48°C for 45'' and 72°C for 4' followed by 25 cycles of 94°C for 30'', 60°C for 30'' and 72°C for 4' and a final step at 68°C for 8'.

[0141] PCR fragments were generated from pBr.Ad35leftITR-pIX, pBr.Ad35.leftITR-pIXΔE1A, pBr.Ad35Δ21K, pBr.Ad35Δ55K1, pBr.Ad35ΔSM and pBr.Ad35ΔE1AΔE1B. All fragments were using the PCR purification kit (Qiagen) according to manufacturer's instructions and final concentrations were estimated on EtBr stained agarose gel using the Eagle Eye II Still Video system and EagleSight software (Stratagene) with the SmartLadder molecular weight marker (Eurogentec) as reference.

[0142] PER.C6 cells were seeded at a density of 2.5×10^6 cells in a T25 culturing flask in DMEM containing 10% fetal calf serum (FCS) and 10mM MgSO₄ and cultured in a humidified stove at 37°C, 10% CO₂. The next day, 3 mg of each of the PCR fragments was cotransfected with 5 μgr NotI digested pWE.Ad35pIX-rITR using LipofectAmine (GIBCO, Life Technologies Inc.) according to the manufacturer's instructions. Two days after the transfection, all cells were passed to a T80 flask and further cultured. Cultures were then monitored for the appearance of CPE. In line with the outcome of previous experiments described in Examples 4 and 6, pBr.Ad35.leftITR-pIX and pBr.Ad35.leftITR-pIXΔE1A showed almost full CPE within one week following transfection. Of the fragments with different E1B deletions, only pBr.Ad35Δ21K showed CPE at the same time as the above two fragments. Constructs pBr.Ad35Δ55K1, pBr.Ad35ΔSM and pBr.Ad35ΔE1AΔE1B did not give CPE at all, not even after harvesting by freeze-thawing and re-infection of the crude lysate onto fresh PER.C6 cells.

[0143] From these experiments, it can be concluded that Ad35-E1B-55K, and not E1B-21K, is necessary for generation and propagation of Ad35-based viruses on Ad5 complementing cell lines. Therefore, Ad35-based viruses having a deletion of the E1A

and E1B-21K genes and having the E1B-55K gene or a functional fragment thereof, can be grown on Ad5 complementing cell lines. Alternatively, Ad35-based viruses can be grown on PER.C6 cells that stably express the full E1B region or the E1B-55K gene, or a functional fragment thereof. The Ad35-E1B-55K gene, or functional parts thereof, may be expressed from a heterologous promoter like, but not limited to, the human PGK promoter, the human cytomegalovirus immediate early promoter (CMV), Rous sarcoma virus promoter, etc., and terminated by a heterologous poly adenylation sequence (pA) like, but not limited to, the hepatitis B virus poly adenylation sequence (HBVpA) and the Simian Virus 40 poly adenylation sequence (SV40pA), etc. As nonlimiting examples, PER.C6 cells that express the Ad35-E1B region driven by the E1B promoter and HBVpA, PER.C6 cells that express the Ad35-E1B region driven by the human PGK promoter and HBVpA and PER.C6 cells that express a functional fragment of Ad35-E1B-55K driven by the human PGK promoter and HBVpA are described below.

Generation of pIG35BL and pIG35BS

[0144] We describe the generation of two expression constructs, pIG.35BS and pIG.35BL, that both carry the Ad35-E1B genes and a neomycin selection marker. The two constructs differ in the length of the fragment containing the E1B promoter. In 35BL, the promoter fragment is longer and includes the 3' end of the E1A region (103 nucl. coding sequence and pA). The E1B region is terminated by the HBVpolyA and the neo^r gene is driven by a hPGK promoter/HBVpA cassette.

[0145] pIG.35BL was made as follows. Construct pRSV.Ad35E1 (described in Example 5, FIG. 9) was digested with NruI and HindIII and the protruding ends were filled in by Klenow treatment. The 7 kb vector fragment was separated from the smaller fragment on gel and isolated using the GENECLAN kit (BIO 101, Inc.). After religation of the DNA and transformation into competent STBL2 cells (Gibco, LTI), correct clones were isolated. pIG.35BL (FIG. 22) contains 273 nucl. upstream of the start site of the E1B-21K coding region.

[0146] pIG.35BS was made in the same way as pIG.35BL except that pRSV.Ad35E1 was digested with NruI and HpaI (both enzymes leave blunt ends),

resulting in a shorter fragment upstream of the coding region of E1B-21K: 97 nucleotides.

[0147] To generate Ad35-E1B expressing cells, PER.C6 cells were seeded in 10 cm dishes at 1×10^6 cells/dish. Two days later, cells were transfected with ScaI linearised constructs. Four dishes were transfected with 1 and four with 2 μ g DNA (total of 16 dishes; Lipofectamine (Gibco, LTI), no carrier DNA used) according to the manufacturer's instructions. The next day, transfected cells received G418-containing medium (0.75 mg/ml). Control transfections using LacZ expression constructs (2 μ g) were stained after 48 hrs and showed a transfection efficiency of ~25%. Four days following addition of selection medium, untransfected cells started to die and again, three days later, clones were becoming visible. A week later, the first clones were picked. Transfection with 1 μ g resulted in less and also, initially, smaller clones (total ~20 clones/dish against >50 clones/dish for the transfection with 2 μ g DNA). The positive control transfection using 2 μ g pcDNA3 (Invitrogen) resulted in ~50 clones.

[0148] In total, 120 clones were picked and 107 were successfully established (55 from pIG35BS and 52 from pIG35BL).

Generation of pIG35Bneo

[0149] pIG35Bneo is an Ad35-E1B expression plasmid from which the E1B genes are expressed from a heterologous promoter (hPGK) and that also contains a neomycin resistance expression cassette. To avoid instability of the plasmid due to recombination events on homologous sequences, the RSV promoter drives the *neo^r* gene. To achieve this, construct pRSVhbv.Neo (described in Example 5, FIG. 12) was digested with ScaI and BamHI and protruding ends were filled in using Klenow enzyme. The 1070 bp fragment containing part of the Ampicilin gene and the RSV promoter was isolated from gel using the GENECLEAN kit (BIO 101, Inc.). Next, pRSVhbvNeo was digested with ScaI and EcoRI, blunted with Klenow and the 3.2 kb fragment containing the *neo* gene, HBVpA, vector and part of the Ampicilin gene was isolated as above. The two fragments were then ligated to give pRSVneo4 (FIG. 23). Construct pIG270 (FIG. 15, described in Example 6) was then digested with EcoRI and NcoI and sticky ends were blunted with Klenow enzyme. The vector-containing fragment was isolated from gel as

described above and religated to give pIG270delE1A. This construct was digested with AvrII and XbaI and protruding ends were filled in using Klenow enzyme. The 2.9 kb fragment containing the hPGK promoter and Ad35-E1B sequences was isolated from gel as above. Next, pRSVneo4 was digested with BglII, blunted with Klenow enzyme, dephosphorylated and isolated from gel. The blunted AvrII/XbaI Ad35-E1B fragment was then ligated with the above prepared pRSVneo4 vector fragment and resulting clones were analysed. One clone that contained both expression cassettes in the same orientation was chosen and named pIG35Bneo (FIG. 24). Detailed analysis of this clone revealed that an extra BglII site was present, probably due to an incomplete Klenow reaction (BglII site at nucl 2949 in FIG. 24).

Generation of pIG35.55K

[0150] Construct pIG35.55K is similar to pIG35Bneo, however, it lacks the coding region of Ad35-E1B-21K. Hereto, both the E1A and E1B-21K sequences are first deleted from pIG270 as follows:

[0151] Construct pIG270 is digested with EcoRI, treated with Klenow enzyme and purified using a PCR purification kit (Qiagen) according to the manufacturer's instructions. The recovered DNA is then digested with AgeI and the ~5 kb vector fragment was isolated from gel as above. Next, Ad35-E1B-55K sequences are amplified by PCR on pIG270 template DNA using the following primers:

35D21: 5'- TTA GAT CCA TGG ATC CCG CAG ACT C-3' (SEQ. I.D. NO. __) and
35B3: 5'- CCT CAG CCC CAT TTC CAG-3' (SEQ. I.D. NO. __).

The conditions used for the amplification are as previously described. The PCR fragment is purified using the PCR purification kit (Qiagen) and digested with NcoI. Following Klenow treatment to fill in the protruding ends, the DNA is further digested with AgeI and again column purified. The thus treated PCR fragment is then cloned into the above prepared EcoRI/AgeI digested vector fragment to give pIG270.ΔE1AΔ21K. The last steps to obtain pIG35.55K (FIG. 25) are equivalent to the last steps described above for the generation of pIG35Bneo, starting with pIG270.ΔE1AΔ21K instead of pIG270.ΔE1A.

[0152] pIG35.55K is then linearized with ScaI and used to transfect PER.C6 cells as described above. Clones that are resistant to G418 selection are picked and analysed for their ability to complement the propagation of E1-deleted Ad35 viruses.

Example 8

New packaging cell lines for the generation and propagation of E1-deleted Ad35-based vectors derived from primary human cells.

[0153] The complete morphological transformation of primary cells by adenovirus E1 genes is the result of the combined activities of the proteins encoded by the E1A and E1B regions. The roles of the different E1 proteins in lytic infection and in transformation have been studied extensively (reviewed in Zantema and van der Eb, 1995; White, 1995, 1996). The adenovirus E1A proteins are essential for transformation of primary cells. The E1A proteins exert this effect through direct interaction with a number of cellular proteins that are involved in regulation of transcription. These include the pRB family of proteins, p300/CBP and TATA binding protein. In addition to this, E1A increases the level of p53 protein in the cells. In the absence of adenovirus E1B activity, the rise in p53 levels leads to the induction of apoptosis. Both proteins encoded by the E1B region counteract the induction of apoptosis, although by different mechanisms. E1B-21K seems to counteract apoptosis in a manner similar to Bcl-2 via interaction with the effector proteins downstream in the apoptosis pathway (Han *et al.*, 1996), whereas E1B-55K functions through direct interaction with p53. Importantly, the molecular mechanism by which the E1B-55K proteins of Ad2 and 5 (subgroup C) and Ad12 (subgroup A) function in the ability to neutralise p53 may differ. Whereas Ad5 E1B-55K binds p53 strongly and the complex localises to the cytoplasm, Ad12-E1B-55K binds p53 weakly and both proteins are localised in the nucleus (Zantema *et al.*, 1985; Grand *et al.*, 1999). Both proteins, however, inhibit the transactivation of other genes by p53 (Yew and Berk, 1992).

[0154] In rodent cells, the activity of E1A, together with either E1B-21K or 55K, is sufficient for full transformation, although expression of both E1B proteins together is twice as efficient (Rao *et al.*, 1992;). In human cells, however, the activity of

the E1B-55K protein seems to be more important, given the observation that E1B-55K is indispensable for the establishment of transformed cells (Gallimore, 1986).

[0155] Example 6 hereof describes the generation of pIG270. In this construct, the Ad35-E1 genes are expressed from the hPGK promoter and transcription is terminated by the HBVpA. The hPGK promoter constitutes a HincII-EcoRI fragment of the promoter sequence described by Singer-Sam *et al.* (1984). The HBVpA is located in a BamHI-BglII fragment of the Hepatitis B virus genome (Simonsen and Levinson, 1983; *see also* Genbank HBV-AF090841). As mentioned before, the promoter and polyadenylation sequences of the E1 expression constructs described in this invention may be derived from other sources without departing from the invention. Also, other functional fragments of the hPGK and HBVpA sequences mentioned above may be used.

[0156] The functionality of pIG270 was shown by transformation of primary Baby Rat Kidney cells (BRK). Comparison with an equivalent Ad5-E1 expression construct taught that Ad35-E1 genes were less efficient in transforming these cells. The same has been found for the E1 genes of Ad12 (Bernards *et al.*, 1982).

[0157] It is unclear which E1 protein(s) determine(s) the difference in transformation efficiency of E1 sequences observed for adenoviruses from different subgroups. In the case of Ad12, transfection studies with chimeric E1A/E1B genes suggested that the efficiency of transformation of BRK cells was determined by the E1A proteins (Bernards *et al.*, 1982). The E1B-55K protein is shown *infra* to contain serotype-specific functions necessary for complementation of E1-deleted adenoviruses. If these functions are related to the regulation of mRNA distribution or another late viral function, it is unlikely that these are involved in the transformation efficiency.

[0158] Analysis of functional domains in the Ad2 or Ad5-E1B-55K proteins using insertion mutants have revealed that functions related to viral replication, late protein synthesis and host protein shut-off are not confined to specific domains but are distributed along the protein (Yew *et al.*, 1990). Using the same set of mutants, the domains important for interaction with p53 and E4-Orf6 were found to be more restricted. In addition to one common binding region (amino acids 262 to 326), p53 binding was affected by mutations at aa 180 and E4-Orf6 binding was affected by mutations at aa 143 (Yew and Berk, 1992; Rubenwolf *et al.*, 1997).

[0159] Altogether, these results indicate that it is difficult to separate the E1B-55K functions related to transformation (p53 binding) and late protein synthesis (Orf6 binding).

[0160] The invention discloses new E1 constructs that combine the high efficiency of transformation of one serotype with the serotype-specific complementation function of another serotype. These new constructs are used to transform primary human embryonic retinoblast cells and human amniocytes.

The generation of pIG535, pIG635 and pIG735

[0161] Construct pIG535 contains the Ad5-E1A region and E1B promoter sequences linked to the Ad35-E1B sequences. Hereto, pIG270 (FIG. 15; see example 6) was digested with EcoRI and NcoI. The 5.3 kb vector fragment was then isolated from gel using the GENECLAN kit (BIO Inc. 101) according to the instructions of the manufacturer. Next, construct pIG.E1A.E1B (FIG. 13; see example 6) was digested with EcoRI and XbaI and the resulting 890 bp fragment was isolated as above. A third fragment was generated by PCR amplification on pIG.E1A.E1B using the following primers:

5E1A-F: 5'- GAG ACG CCC GAC ATC ACC TG -3' (SEQ. I.D. NO. __) and

5E1B-R: 5'- CAA GCC TCC ATG GGG TCA GAT GTA AC -3' (SEQ. I.D. NO. __).

The following PCR program was used: 94°C for 2' followed by 30 cycles of 94°C for 30'', 60 °C for 30'' and 72 °C for 1', and a final step at 72°C for 10' to ensure blunt ends.

[0162] The resulting 400 bp PCR fragment was digested with XbaI and NcoI. After gel isolation as above, the three fragments were ligated and transformed into STBL-2 bacteria. One colony containing all three fragments in the correct order was selected and designated pIG535 (FIG. 26).

[0163] Construct pIG635 contains the Ad5-E1A and a chimeric Ad5-Ad35-E1B region such that the 21K sequence is essentially from Ad5 and linked to the Ad35-E1B-55K sequences as far as not overlapping with the 21K sequences. First, part of the Ad5-E1 sequences are amplified by PCR using pIG.E1A.E1B as template and the following primers:

5AK: 5'- GAG CGA AGA AAC CCA TCT GAG -3' (SEQ. I.D. NO. __) and

2155R: 5'- GGT CCA GGC CGG CTC TCG G -3' (SEQ. I.D. NO. ____). Amplification is accomplished with Pwo DNA polymerase (Roche) according to manufacturer's instructions. The 210 bp fragments is then purified from the primer sequences using the PCR purification kit (Qiagen).

[0164] A second PCR fragment is amplified from pIG270 DNA as described above but with the following primers:

2155F: 5'- CCG AGA GCC GGC CTG GAC -3' (SEQ. I.D. NO. ____)

35F10: 5'- GCT CTA GAC CTG CAG GTT AGT CAG TTT CTT CTC CAC TG -3' (SEQ. I.D. NO. ____).

[0165] The 1.3 kb amplified fragment is purified as above and mixed in a 1:1 molar ratio with the first PCR fragment. The mixture is then first subjected to a PCR reaction without the addition of primers using Pwo DNA polymerase and the following program: 94 °C for 2' and then 5 cycles of 94°C for 30'', 60 °C for 30'', 72 °C for 90''. Subsequently, primers 5AK and 35F10 are added at 0.6 µM concentration after which a last PCR amplifies a 1.5 kb fragment. Hereto, temperature was set as follows: 94 °C for 2', then 30 cycles of 94 °C for 30'', 60°C for 30'' and 72 °C for 90'', followed by a final step at 72°C for 10' to ensure blunt ends. The resulting product is purified using the PCR purification kit (Qiagen) as above and digested with KpnI and SbfI (isoschizomer of Sse8387I). The digested DNA is then isolated from gel using the GENECLAN kit (BIO Inc., 101). Construct pIG.E1A.E1B is digested with KpnI and SbfI and the vector-containing fragment is isolated from gel as above. This fragment is ligated to the above prepared final PCR product and the ligation mixture is transformed into STBL-2 cells (Gibco, LTI) according to manufacturer's instructions. This gives construct pIG635 (Fig. 27).

[0166] In construct pIG735, the border between Ad5 derived sequences and Ad35 derived sequences is located more 3' than in construct pIG635. First, a BspEI site is introduced in the Ad5 sequence of construct pIG.E1A.E1B without changing the amino acid sequence. Hereto, Ad5 sequences from pIG.E1A.E1B are amplified using the following PCR primers:

[0167] 5AK: see above, and Bsp-R: 5'- GCT CTA GAC CTG CAG GGT AGC AAC AAT TCC GGA TAT TTA CAA G -3' (SEQ. I.D. NO. ____). Amplification is

accomplished using Pwo DNA polymerase (Roche) according to the manufacturer's instruction. The following PCR program is used: 94°C for 2' followed by 30 cycles of 94°C for 30'', 60 °C for 30'' and 72 °C for 30'', and a final step at 72°C for 10' to ensure blunt ends. The resulting 0.6 kb fragment is purified as above and digested with KpnI and SbfI and ligated to the above described KpnI/SbfI digested pIG.E1A.E1B vector fragment. Selection of colonies after transformation of STBL-2 bacteria (Life Techn. Inc.) gives construct pIG.E1Δ55K. pIG.E1Δ55K is then digested with SbfI and partially with BspEI. The 6.4 kb SbfI-partial BspEI digested vector fragment is then isolated from gel using the geneclon kit (BIO 101, Inc.). Next, pIG270 is digested with BspEI and SbfI and the resulting 915 bp fragment is isolated from gel as above. This fragment is then ligated to the above prepared SbfI/partial BspEI digested pIG.E1Δ55K vector fragment and transformed into STBL-2 competent cells. This gives construct pIG735 (FIG. 28). Clones are analysed by restriction enzyme digestion and sequencing to ensure correct ligation of the fragments. Constructs pIG535, pIG635 and pIG735 can be used to generate complementing cell lines from primary human cells as described in Example 6.

Example 9

PER.C6-based complementing cell lines for E1-deleted Ad35 viruses.

[0168] PER.C6 cells were seeded in 10 cm culture dishes at a density of 3×10^5 cells/dish in DMEM (Gibco BRL) complemented with FBS (Gibco BRL) up to 10% and 10mM $MgCl_2$ (4.9 M stock solution, Sigma). Two days later, 9 dishes were transfected with 1 μ g ScaI linearised pIG35.55K DNA (see example 7) and 9 dishes were transfected with 1.5 μ g ScaI linearised pIG35.55K DNA. Separate control dishes were transfected with 1 or 1.5 μ g ScaI linearised pAdApt35.LacZ to monitor transfection efficiency and with 1 or 1.5 μ g ScaI linearised pcDNA.nlsLacZ. pcDNA.nlsLacZ is a pcDNA3-based plasmid (Invitrogen) with the nlsLacZ gene (Bonnerot et al., 1987) driven by the CMV promoter. pcDNA.nlsLacZ also contains a neo^r expression cassette. As a negative control one extra dish was transfected with linearised pAdApt35.LacZ, a construct that lacks the neo^r selection gene. All transfections were performed with the LipofectAmine transfection kit (Invitrogen/Life Technologies) according to

manufacturers instructions using 5 ml LipofectAmine reagent/ μ g DNA. Cells were incubated for 4 hrs with the transfection mixture after which the medium was replaced with PER.C6 culture medium. The next day medium was replaced with culture medium containing 0.5 mg/ml G418 (Gibco BRL) except in the two dishes that were transfected with 1 or 1.5 μ g pAdApt35.LacZ. These latter dishes were used to monitor LacZ expression two days following transfection. After X-gal staining of these cultures transfection efficiency was estimated at approximately 40% with slightly more blue cells in the dish transfected with 1.5 μ g DNA. Selection medium was refreshed twice weekly in the remaining transfected dishes. Within two weeks following first addition of selection medium most cells in the negative control dish (transfected with 1.5 μ g pAdApt35.LacZ) were dead. In the dishes transfected with pcDNA.nlsLacZ cell clones were becoming visible. Since the cells transfected with pIG35.55K seemed to be more resistant to G418, the concentration was raised to 0.75 mg/ml 3 weeks following transfection. Three days and seven days later a total of 196 cell clones were picked from the dishes transfected with pIG35.55K and seeded in separate wells of 96-well plates.

[0169] Cells remaining after colony picking of two 10 cm dishes of the transfection with 1 μ g pIG35.55K DNA were trypsinised, pooled and expanded to give pool PER55K(1.0) The same was done for two dishes of the 1.5 μ g transfection. The PER55K(1.0) cell pool was expanded and seeded in 4 T25 flasks at a density of 3.5×10^4 cells/flask for transfection to test virus generation. In addition, 3 T25 flasks with parental PER.C6 cells were seeded at the same density. pAdApt35.eGFP (an adapter plasmid containing the green fluorescent protein as marker gene; see example 4) was digested with PacI to liberate the adenoviral sequences from the plasmid backbone. pWE.Ad35.pIX-rITR (see example 4) was digested with NotI to liberate the adenoviral sequences from the cosmid backbone. 2 flasks with PER.C6 cells and 2 flasks with PER55K(1.0) cells were transfected with 2 μ g digested pAdApt35.eGFP and 6 μ g digested pWE.Ad35.pIX-rITR each. One flask of each cell line was transfected with 8 μ g pAdApt35.LacZ to monitor transfection efficiency. The remaining flask with PER55K(1.0) cells served as a negative control and was treated as the others but did not receive the transfection mixture. All transfections were performed with LipofectAmine (Invitrogen/Life Techn.) according to manufacturers instructions using for each

transfection a total of 8 µg DNA and 40 µl LipofectAmine reagent. The transfection mixture was removed after 4 hrs incubation and fresh culture medium was added. Transfections were done the day after seeding of the cells and again two days later cells in the T25 flasks were transferred to a T80 flask except for the LacZ control transfections. These were stained with X-gal solution after mild fixation. After five hours incubation with staining solution, the percentage of blue cells was estimated at approximately 90% in both flasks showing that transfection went well for both cell lines. Four days following the passage to the T80 flasks the transfected PER55K(1.0) cultures showed starting CPE (cytopathogenic effect, indicative of virus replication) with approximately 100 events/flask. The untransfected PER55K(1.0) cells were grown confluent with no evidence of CPE. In the transfected PER.C6 cultures only three CPE events were visible in the confluent monolayer of cells. Again three days later, the transfected PER55K(1.0) cultures showed full CPE, with all cells rounded and detached in clumps. In contrast, in the PER.C6 cultures the few events of CPE had not progressed and cells were still in monolayer. This confirms earlier observations that generation of E1-deleted Ad35-based viruses on PER.C6 is very inefficient. Also the untransfected PER55K(1.0) cultures showed, as expected, a confluent monolayer with no CPE. The cells and medium in the PER55K(1.0) flasks with full CPE were harvested and subjected to two freeze/thaw cycles after which the cell debris was removed by centrifugation at 3000 rpm for 10 minutes in a table centrifuge. One of the resulting crude lysates was used to infect a fresh culture of PER55K(1.0) cells in a T175 flask (1.5 ml/flask). Cells and medium were harvested at full CPE four days later. This shows that infectious virus had formed in the initial transfections. GFP expression was confirmed by fluorescent microscopy of A549 cells infected with the crude lysate. The crude lysate was then used to analyse complementation of this E1-deleted Ad35.AdApt.eGFP virus in the individual clones as described below.

[0170] The above-described clones that were picked from the pIG35.55K transfected PER.C6 cells were expanded and were functionally tested for the ability to sustain replication of Ad35.AdApt.eGFP. Hereto, the clones were seeded at two densities in 6-well plates and one day later infected with 15 ml of the above described crude lysate. CPE was monitored the day after. Of the 146 clones tested in this way 19 gave full CPE

at day 2 or 3 and 68 gave full CPE at day 5 or 6. The remaining clones had only partial CPE or showed a few non-progressing events. The latter were indistinguishable from PER.C6 cells that were taken along as a negative control.

[0171] Based on these results a selection of 24 clones was made that were further screened for the ability to generate recombinant E1-deleted viruses following transfection of the pAdApt35.GFP adapter plasmid and the large pWE.Ad35.pIX-rITR cosmid clone. Hereto, clones were plated in T25 flasks and transfected with 2 µg of the adapter and 6 µg of the backbone plasmid using LipofectAmine as described above. Two days following the transfection, cells were transferred to T80 flasks to prevent overconfluency of the cultures. Of the 24 clones 5 gave full CPE three days after passage to T80 and another 13 clones gave progressing to full CPE the day after. The remaining 6 clones showed no CPE or only starting. In comparison: routine generation of E1-deleted Ad5 vectors on PER.C6 cells generally results in full CPE four to six days after transfer to T80 flasks.

[0172] This shows that the new clones efficiently complement E1-deleted adenovirus vectors. One of the clones (clone #16) described above was used to generate and produce multiple batches of E1 and E1/E3 deleted Ad35 viruses containing different transgenes. Hereto, virus in crude lysates resulting from transfections as described above, but using different adapter plasmids, were plaque purified on the new cell line. Single plaques were tested for transgene activity and then amplified for medium scale production in 4-8 triple layer flasks (3x175 cm/flask). Cells were harvested at full CPE and the virus was released and purified as routinely done for adenoviruses and described in example 1. The extraction step with freon to remove cellular debris was, however, replaced by a centrifugation step. Thus after incubation with DnaseI, the cell debris was centrifugated in conical 50 ml tubes (Greiner) at 8000 rpm in a table top centrifuge (Beckman Coulter Allegra 21R with fixed angle rotor) for 30 minutes at 4°C. This step is repeated in a fresh 50 ml tube untill the supernatant was clear (usually one time). The amount of virus particles was determined by HPLC (Shabram et al., 1997). Table IV presents the yields after downstream processing of medium scale productions of E1- and E1/E3-deleted Ad35 viruses on triple layer flasks with PER55K clone #16 cells. The

amount of purified virus particles is comparable with the yields of Ad5-based vectors on PER.C6 cells.

[0173] We conclude that we have generated multiple cell lines that efficiently complement fully E1-deleted Ad35-based vectors. Thus, Ad35 E1B-55K expression in an Ad5 complementing cell line facilitates replication of Ad35 vectors.

Example 10

New complementing cell lines from primary cells.

[0174] Example 8 described the generation of construct pIG535, a hybrid Ad5E1A-Ad35 E1B expression plasmid. pCC536s and pIG536 are also hybrid Ad5-Ad35 E1 constructs but with the E1A region, E1B promoter and most of the E1B-19K gene derived from Ad5 and most of the E1B-55K gene derived from Ad35. Constructs pCC536s and pIG536 differ only in the heterologous poly adenylation sequence that terminates the E1B transcript: pIG536 has the HBV pA sequence and pCC536s has a synthetic pA sequence (SpA). The SpA sequence consists of the upstream sequence element (USE) of the human C2 complement gene (Moreira et al., 1995) and the synthetic pA sequence (SPA) described by Levitt et al., 1989.

[0175] The synthetic polyA sequence is build up using the following oligo's:
C2SPA-1: 5'- CCC TGC AGG GAC TTG ACT CAT GCT TGT TTC ACT TTC ACA TGG AAT TTC CCA GTT ATG AAA TTA ATA AAG -3'
C2SPA-2: 5'- GTC TAG ACA CAC AAA AAA CCA ACA CAC TAT TGC AAT GAA AAT AAA TTT CCT TTA TTA ATT TCA TAA CTG -3'

Oligonucleotides were mixed at 10μM concentration in 1x annealing buffer (10mM Tris HCl pH 7.5, 100mM NaCl, 1mM EDTA) and, using a PCR machine, the solution was heated to 94°C for 5 minutes and then cooled down to 65°C at 0.5°C / second and after incubation at 65 °C for 5 minutes further cooled down to 20°C at 0.05 °C / second. Subsequently, 10 μl 2mM dNTPs, 0.5 μl 1M MgCl₂ and 3 μl Klenow fragment (New England Biolabs) was added to 87 μl of the annealed sample and the mixture was incubated at room temperature for 30 minutes. 1 μl of the annealed and Klenow treated sample was then amplified using the following primers:

C2for: 5'- CGG GAT CCC CTG CAG GGA CTT GAC -3'

and

SPArev: 5'- TTG CGA CTT AAG TCT AGA CAC ACA AAA AAC C -3' using Pwo DNA polymerase (Roche) according to manufacturers instructions but with addition of DMSO (Sigma) to a final concentration of 3%. The PCR program was set at 94°C for 2 minutes, followed by 30 cycles of (94 °C for 30'', 55°C for 30'' and 72°C for 20''). Where in this document PCR programs are described ' means time in minutes and '' means time in seconds. The amplified DNA was then purified using the QIAquick PCR purification kit (Qiagen) and digested with XbaI and SbfI. The digested product was then again purified with the PCR purification kit to remove the small digested ends. Construct pIG270 was also digested with XbaI and SbfI (isoschizomer of Sse8387I) and the resulting 5.9 kb vector containing fragment was isolated from gel using the GeneClean II kit (Bio101, Inc). The treated vector and PCR insert were then ligated to give pCC271 (Figure 29). pCC271 thus contains the PGK promoter, the Ad35 E1 region (nucl. 468 to and including 3400 from Ad35 sequence in example 3 and figure 5) and the synthetic pA (SpA). The synthetic pA sequence was then also cloned into the construct pIG535 as follows.

[0176] pIG535 was digested with EcoRI, PstI and ScaI (All enzymes from New England Biolabs digested in NEB buffer 3) and the 3 kb insert corresponding to chimeric Ad5-Ad35 E1 region was purified using the GeneClean II kit (Bio 101, Inc.). Construct pCC271 was digested with EcoRI and PstI and the 3 kb vector fragment containing the SpA and PGK promoter was isolated as above. Both isolated fragments were ligated and transformed into STBL-2 competent cells (Invitrogen/LifeTechnologies) to give pCC535s (Figure 30). pCC535s contains the same Ad5-Ad35 E1 sequences as pIG535 however, a different pA sequence.

[0177] For the construction of pCC536s, a subclone was made with the new hybrid E1B sequences. Hereto, Ad5 E1A/E1B21K sequences were amplified using the primers

5AK: 5'- GAG CGA AGA AAC CCA TCT GAG- 3' and

2155R: 5'- GGT CCA GGC CGG CTC TCG G-3' with pIG.E1A.E1B (see example 6 and Figure 13) as template DNA using Pwo DNA polymerase (Roche) according to manufacturers instructions and in addition a final concentration of 3% DMSO. The

program was set at: 94°C for 2' followed by 30 cycles of (94°C for 30'', 58°C for 30'' and 72°C for 30'') and ended with 68°C for 8'. This resulted in a 210 bp fragment corresponding to nucl. 2022-2233 of the Ad5 sequence. A second PCR was performed on pCC271 with primers

2155F: 5'- CCG AGA GCC GGC CTG GAC C-3' and

35F10: 5'- GCT CTA GAC CTG CAG GTT AGT CAG TTT CTT CTC CAC TG-3'.

[0178] The same PCR program was used but now with an elongation time of 90''. The resulting 1.3 kb fragment corresponds to nucl. 2112 to 3400 of the Ad35 sequence with an SbfI site at the 3'end. Note that primers 2155F and 2155R are fully complementary allowing assembly of the two fragments as follows:

[0179] Both PCR fragments were purified from gel using the Qiagen gel extraction kit. Aliquots of the purified samples were then mixed in equimolar ratio and used as template for an assembly PCR amplification with primers 5AK and 35F10 with Pwo DNA polymerase as above using the program settings:

94°C for 2', and 5 cycles of (94°C for 30'', 60°C for 30'' and 72°C for 2') followed by 25 cycles of (94°C for 30'', 58°C for 30'' and 72 °C for 90''). The resulting 1.5 kb fragment was purified from gel using the QIAquick gel extraction kit (Qiagen), ligated to the pCR-Script/Amp cloning vector (Stratagene) and transformed into DH5a competent cells (Invitrogen/Life Technologies) resulting in pCR535E1B (Figure 31). This construct was checked by restriction analysis and sequencing to confirm correct amplification of target sequences.

[0180] pCR535E1B was then digested with NotI and protruding ends were made blunt with Klenow fragment. The DNA was then purified using the QIAquick PCR purification kit (Qiagen) and eluted DNA was digested with PstI. The 1.5 kb fragment containing the chimeric E1 sequences from the pCR535E1B vector was purified from gel using the GeneClean II kit (Bio101, Inc.). This fragment was ligated to vector pCC535s digested with PvuII and PstI, and transformed into STBL-2 competent cells (Invitrogen/Life Technologies) to give pCC2155s (Figure 32). To complete the pCC536s construct Ad5-E1 sequences were then cloned into the pCC2155s subclone. Hereto, pIG.E1A.E1B was digested with EcoRI and KpnI and the 1.6 kb fragment corresponding to Ad5 E1A and Ad5 E1B 21K (nucl. 459 to 2048 of the Ad5 sequence) was isolated

from gel using the GeneClean kit. pCC2155s was digested with EcoRI and KpnI and the vector containing fragment was also gel purified. Ligation of both isolated fragments and transformation into DH10B electrocompetent cells (Invitrogen/Life Technologies) resulted in pCC536s (Figure 33). The hybrid E1B sequences are shown in Figure 38 in more detail. FIG38A shows an alignment of protein sequences of E1B-21K in the pCC536s construct with wild type (wt) Ad35 and Ad5 sequences. As can be seen most of the E1B-21K protein in pCC536s is derived from Ad5 except for the C-terminal 6 amino acids that are identical to Ad35 E1B-21K. Figure 38B shows the same alignment for the E1B-55K proteins. In this case the N-terminal amino acids of pCC536s are identical to Ad5 upto aa 65. The remainder is identical to Ad35 E1B-55K. Obviously, different hybrid E1B-55K constructs can be designed using the general method outlined above without departing from the invention.

[0181] Construct pIG536 was made by replacing a fragment with the SpA in pCC536s with the corresponding fragment from pIG270 (example 6, Figure 15) containing the HBVpA. Hereto, pIG270 was digested with BamHI and BglII and the 1.8 kb insert was isolated from gel using the GeneClean II kit (Bio 101, Inc.). pCC536s was digested with the same enzymes and the 4.8 kb vector containing fragment was purified from gel as above. Ligation of both isolated fragments and transformation into STBL-2 competent cells (Invitrogen/Life Technologies) gave construct pIG536 (Figure 34).

[0182] The generated E1 constructs were tested in primary baby rat kidney (BRK) cells as described in example 6. The results (Table V) confirm earlier observations that Ad5-E1 genes more efficiently transform primary BRK cells than Ad35 E1 genes. The chimeric Ad5-Ad35 E1 expression constructs, pCC535s and pCC536s, produced more transformed colonies than the full Ad35 E1 constructs, pIG270 and pCC271. Furthermore, the use of a synthetic poly adenylation sequence in pCC535s resulted in slightly more foci compared to the HBVpA variant pIG535.

[0183] Human embryonic retinoblast (HER) cells were isolated from the eyes of aborted fetuses of 18 and 21 weeks of age. The eyes were brought in a 6 cm dish with PBS and cleared from outside tissue. An incision was made to reach the inner side and the gray cell layer at the inner back of the eyes containing the retinoblasts, was scraped off. This layer was transferred to a 14 ml tube in 2ml of PBS and tissue was allowed to

sediment after which the PBS was removed. 2 ml trypsin (0.25%, no EDTA, GibcoBRL) was added and incubated for 5 minutes at 37°C with occasional swirling. Tissue pieces were allowed to sediment and 1 ml trypsin with cells was transferred to a new tube. To this tube 4 ml culture medium (DMEM with 10% FCS) was added and the tube was stored on ice. The remaining tissue pieces in trypsin were brought in a 6 cm dish and cut into smaller pieces. These were, after addition of 2 ml fresh trypsin, again incubated in a 14 ml tube at 37°C with occasionally swirling. Then this mixture was added to the first isolated cells in culture medium and the total was centrifugated at 1000 rpm in a table top centrifuge. Supernatant was removed and cells were resuspended in 10 ml of culture medium. The isolated HER cells were plated in two 6 cm dishes and incubated at 37°C/10% CO₂. Upon 90% confluency cultures were split 1:3 and further incubated. This procedure was repeated until enough dishes were obtained to be used for transfection and further culturing. Transfections were performed at different passage numbers using the CaPO₄ cotransfection kit (Invitrogen/Life Technologies) according to manufacturers instructions. For each dish (50-70% confluency) 20 µg DNA was used. Initial transfections were performed with pIG.E1A.E1B, an Ad5-E1 expression construct, and with pIG535, the hybrid Ad5-E1A/Ad35-E1B expression construct. 2-3 weeks following transfection transformed foci became visible in the pIG.E1A.E1B transfected dishes. On average 15-20 foci/dish were found in the dishes that were transfected with pIG.E1A.E1B. Over 30 clones were picked and transferred to 96-well plates. Upon confluency cells were passaged to larger culture plates or flasks and finally viable frozen in ampoules in liqN₂ from a T175 flask. All picked clones were established in this way. Transformed foci appeared much later in the dishes that were transfected with pIG535, the first around five weeks following transfection. On average 3-4 clones were found per dish. A total of 46 clones were picked from 7 weeks to 3 months after transfections of which 14 were viable and could be passaged multiple times. Of these, 2 clones (clone #45 and #75) were grown up to a T175 flask and viable frozen in ampoules in liqN₂.

[0184] Primary HER cells were also transfected with constructs pCC535s and pCC536s. Transfection of pCC535s let to an average of 2 clones/dish and a total of 50 clones were picked. Of these picked clones 2 could be established. From the transfection with pCC536s, at least one clone could be established.

[0185] The above-described experiments show that primary HER cells can be transformed with hybrid Ad5-Ad35 E1 sequences. The efficiency of transformation was lower than obtained with the complete Ad5 E1 region. We then tested whether the new cell lines could complement recombinant Ad35-based E1-deleted vectors. Hereto, the clone #45 that was obtained from the pIG535 transfection was seeded in T25 flasks at a density of 7×10^6 cells/flask and infected with Ad35.AdApt.eGFP virus (see example 9) at a multiplicity of infection (moi) of 5 and 25 virus particles/cell. Full CPE was seen at days 4 and 5 for the moi 25 and 5 respectively. As a comparison parallel cultures of clone #45 cells that were infected with Ad5.AdApt.eGFP viruses gave full CPE at days 7 and 8 for moi 25 and 5 respectively. The initial infection efficiency was comparable for Ad5 and Ad35 viruses, ~80% (moi=5) and ~95% (moi=25) of the cells were infected with GFP virus one day following infection as measured by fluorescence microscopy. Cells from clone #75 were seeded in a 6-well plate at a density of 2×10^6 cells/well and infected with Ad35.AdApt.eGFP or Ad5.AdApt.eGFP at moi 5 (VP/cell). Again initial infection efficiency was comparable for both viruses. Full CPE was observed at day 4 in case of Ad35.AdApt.eGFP infection whereas Ad5.AdApt.eGFP infected clone #75 cells gave full CPE on day 7. The difference in replication efficiency on Ad35 complementing cells between Ad35 and Ad5 recombinant vectors is even more clear when virus is generated by plasmid transfection. This is exemplified by the following transfection experiment. Clone #45 cells were seeded in T25 flasks at a density of 3.5×10^6 cells and transfected three days later using LipofectAmine reagent (Invitrogen/Life Technologies) according to manufacturers instructions and described above. 2 μ g pAdApt35.eGFP adapter plasmid digested with PacI was cotransfected with 6 μ g pWE.Ad35.pIX-rITR or pWE.Ad35.pIX-rITRAE3 backbone cosmid digested with NotI. 2 μ g pAdApt.eGFP (Ad5 adapter plasmid, described in WO 00/70071) digested with PacI was cotransfected with 6 μ g pWE.Ad5.AflII-rITRsp (Ad5 backbone plasmid, described in WO 00/70071) also digested with PacI. One T25 was not transfected and served as a negative control. One day later transfection efficiencies were monitored by fluorescent microscopy and estimated at 10-15% in all eGFP transfections. Three days following transfection cells were transferred to T80 flasks and further incubated at 37°C/10%CO₂. Again three days later CPE events were becoming visible in the cultures transfected with the

pAdApt35.eGFP and the pWE.Ad35pIX-rITR+ or – E3. The transfections with the E3-deleted backbone contained more green fluorescent cells and more CPE events. The transfection with Ad5 plasmids showed only around 20% green fluorescent cells, of which most were dying, and no CPE events. Two days later this difference had become bigger since cultures transfected with the pAdApt35.eGFP and the pWE.Ad35pIX-rITRΔE3 clearly showed 80% CPE and cultures transfected with the pAdApt35.eGFP and the pWE.Ad35pIX-rITR constructs showed progressing CPE events. The Ad5 transfected culture did not show any progression. Table VI summarizes these results.

[0186] We conclude that the new complementing cell lines described above efficiently sustain replication of E1 deleted Ad35-based viruses and that the generation and replication of E1 deleted Ad5-based viruses is less efficient. Apparently, also Ad35-E1B55K proteins do not form a functional complex with Ad5-E4Orf6 proteins. Thus the serotype specificity for complementation is now also shown for recombinant Ad5 vectors on Ad35 packaging cells.

Example 11

Generation of pWE.Ad.pIX-rITRΔE3.

[0187] The early region-3 of human adenoviruses contains multiple coding regions for proteins that interfere with the host immune response to adenoviral infection. When adenoviral vectors are used as vaccine carrier such interference is unwanted. Therefore, we constructed an Ad35 backbone cosmid lacking the E3 region.

[0188] Hereto, construct pBr.Ad35.PRn (Figure 35; described in example 13 in publication EP 1 054 064 A1) was digested with *Stu*I and *Mlu*I and the 17.3 kb vector fragment was purified from low melting point (LMP) gel using agarase enzyme (Roche) according to manufacturers instructions. Next, a PCR fragment was generated on pBr.Ad35.PRn using primers:

35E3for: 5'- AAT GAC TAA TGC AGG TGC GC-3' and

35E3rev: 5'- CGA CGC GTT GTA GTC GTT GAG CTT CTA G-3'. For the amplification Pwo DNA polymerase (Roche) was used according to manufacturers instructions and program set at: 94°C for 2', 30 cycles of (94°C for 30'', 58°C for 30'' and 72°C for 1') and a final incubation at 68°C for 8'. The 833 bp PCR product was

purified using the QIAquick PCR purification kit (Qiagen) and digested with MluI and StuI. The digested DNA was purified from gel using the QIAquick gel extraction kit (Qiagen). Both isolated fragments were ligated and transformed into DH5a competent cells (Invitrogen/Life Technologies) to give pBr.Ad35.PRnΔE3 (Figure 36). The plasmid was checked by restriction analysis and sequencing of the PCR amplified insert. The E3 deletion was then cloned into the pWE.Ad35.pIX-rITR cosmid backbone. Hereto, pWE.Ad35.pIX-rITR (see example 4 and Figure 8) was digested with PacI and the DNA was purified by precipitation with isopropanol and washing with 70% EtOH. Following resuspension in milliQ water, the DNA was digested with SwaI and the 22.8 kb vector containing fragment was purified from LMP gel using agarase enzyme as above. Construct pBr.Ad35.PRnΔE3 was digested with PacI and SwaI in the same manner and the 16.6 kb fragment was also isolated using agarase enzyme. Both isolated fragments were ligated using 0.5-0.6 μg of each fragment. Ligated fragments were then packaged using λ-phage packaging extracts (Stratagene) according to manufacturers instructions and mixed with STBL-2 cells. Bacteria were plated on LB+Amp plates and resulting colonies were analyzed for the presence of the correct construct. This gave construct pWE.Ad35.pIX-rITRΔE3 (Figure 37). The E3 deletion extends from nucl. 27648 to 30320 of the Ad35 sequence (example 3) and thus spans a 2.6 kb region.

[0189] Cotransfection of NotI digested pWE.Ad35.pIX-rITRΔE3 and pIPsp-1 digested pAdApt35.eGFP onto PER55-clone #16 cells (see example 9) as described above gave rise to GFP expressing Ad35-based viruses. Upon isolation of viral DNA from these viruses, PCR amplification of the E3 region showed that the viruses were deleted for 2.6 kb of E3 sequences as expected.

Table I:

Serotype	Elution [NaCl] mM	VP/ml	CCID50	log ₁₀ VP/CCID50 ratio
1	597	8.66x10 ¹⁰	5.00x10 ⁷	3.2
2	574	1.04x10 ¹²	3.66x10 ¹¹	0.4
3	131	1.19x10 ¹¹	1.28x10 ⁷	4.0
4	260	4.84x10 ¹¹	2.50x10 ⁸	3.3
5	533	5.40x10 ¹¹	1.12x10 ¹⁰	1.7
6	477	1.05x10 ¹²	2.14x10 ¹⁰	1.7
7	328	1.68x10 ¹²	2.73x10 ⁹	2.4
9	379	4.99x10 ¹¹	3.75x10 ⁷	4.1
10	387	8.32x10 ¹²	1.12x10 ⁹	3.9
12	305	3.64x10 ¹¹	1.46x10 ⁷	4.4
13	231	4.37x10 ¹²	7.31x10 ⁸	3.8
15	443	5.33x10 ¹²	1.25x10 ⁹	3.6
16	312	1.75x10 ¹²	5.59x10 ⁸	3.5
17	478	1.39x10 ¹²	1.45x10 ⁹	3.0
19	430	8.44x10 ¹¹	8.55x10 ⁷	4.0
20	156	1.41x10 ¹¹	1.68x10 ⁷	3.9
21	437	3.21x10 ¹¹	1.12x10 ⁸	3.5
22	365	1.43x10 ¹²	5.59x10 ⁷	3.4
23	132	2.33x10 ¹¹	1.57x10 ⁷	4.2
24	405	5.12x10 ¹²	4.27x10 ⁸	4.1
25	405	7.24x10 ¹¹	5.59x10 ⁷	4.1
26	356	1.13x10 ¹²	1.12x10 ⁸	4.0
27	342	2.00x10 ¹²	1.28x10 ⁸	4.2
28	347	2.77x10 ¹²	5.00x10 ⁷	4.7
29	386	2.78x10 ¹¹	2.00x10 ⁷	4.1
30	409	1.33x10 ¹²	5.59x10 ⁸	3.4
31	303	8.48x10 ¹⁰	2.19x10 ⁷	3.6
33	302	1.02x10 ¹²	1.12x10 ⁷	5.0
34	425	1.08x10 ¹²	1.63x10 ¹¹	0.8
35	446	3.26x10 ¹²	1.25x10 ¹¹	1.4
36	325	9.26x10 ¹²	3.62x10 ⁹	3.4
37	257	5.86x10 ¹²	2.8x10 ⁹	3.3
38	337	3.61x10 ¹²	5.59x10 ⁷	4.8
39	241	3.34x10 ¹¹	1.17x10 ⁷	4.5

Continued on next page.

Serotype #	Elution [NaCl] mM	VP/ml	CCID50	log ₁₀ VP/CCID50 ratio
42	370	1.95x10 ¹²	1.12x10 ⁸	4.2
43	284	2.42x10 ¹²	1.81x10 ⁸	4.1
44	295	8.45x10 ¹¹	2.00x10 ⁷	4.6
45	283	5.20x10 ¹¹	2.99x10 ⁷	4.2
46	282	9.73x10 ¹²	2.50x10 ⁸	4.6
47	271	5.69x10 ¹¹	3.42x10 ⁷	4.2
48	264	1.68x10 ¹²	9.56x10 ⁸	3.3
49	332	2.20x10 ¹²	8.55x10 ⁷	4.4
50	459	7.38x10 ¹²	2.80x10 ⁹	3.4
51	450	8.41x10 ¹¹	1.88x10 ⁸	3.7

Legend to Table I:

All human adenoviruses used in the neutralization experiments were produced on PER.C6 cells (Fallaux *et al.*, 1998) and purified on CsCl as described in example 1. The NaCl concentration at which the different serotypes eluted from the HPLC column is shown. Virus particles/ml (VP/ml) were calculated from an Ad5 standard. The titer in the experiment (CCID50) was determined on PER.C6 cells as described in Example 1 by titrations performed in parallel with the neutralization experiment. The CCID50 is shown for the 44 viruses used in this study and reflects the dilution of the virus needed to obtain CPE in 50% of the wells after 5 days. The ratio of VP/CCID50 is depicted in log₁₀ and is a measurement of the infectivity of the different batches on PER.C6 cells .

Table II. AdApt35.LacZ viruses escape neutralization by human serum.

Virus	Human serum dilution					
	no serum	10x	50x	250x	1250x	6250x
AdApt5.LacZ moi: 5 VP/cell	100 %	0 %	0 %	1 %	40 %	80 %
AdApt35.LacZ 250 µl crude lysate	100 %	100 %	100 %	100 %	100 %	100 %

Table III: The numbers of foci obtained with the different E1 expression constructs in BRK transformation experiments.
Average # of foci/dish:

	Construct	1 µgr	5 µgr
Experiment 1	pIG.E1A.E1B	nd	60
	pIG.E1A.E1B	nd	35
	pRSVAd35E1	0	3
	pIG.Ad35.E1	3	7
Experiment 2	pIG.E1A.E1B	37	nd
	pIG.Ad35.E1	nd	2
Experiment 3	pIG.E1A.E1B	nd	140
	pIG.Ad35.E1	nd	20
	pIG270	nd	30

Table IV: Yields of E1- and E1/E3- deleted Ad35 viruses on clone #16 cells produced on triple layer flasks.

Virus	Scale(T175III flasks)	Total # of Virus Particles after DSP	VP/cell
Ad35.AdApt.eGFP	4	7.5×10^{11}	2500
Ad35.ΔE3.AdApt.empty	8	2×10^{12}	3300
Ad35.ΔE3.AdApt.LacZ	8	3.8×10^{11}	600
Ad35.ΔE3.AdApt.MV-F	4	8.8×10^{11}	2900
Ad35.ΔE3.AdApt.MV-H	8	2.6×10^{12}	4250

Table V: Transformation efficiencies on BRK cells with different Ad-E1 expression constructs.

	Construct	Transfected DNA (μ g)	# foci per dish
Experiment 1	pIG.E1A.E1B	5	44
	pIG270	5	0
	pCC271	5	0
	pIG535	5	1
	pCC535s	5	2.5
Experiment 2	pIG.E1A.E1B	4	15
	pCC271	4	0
	pCC535s	4	3
	pCC536s	4	3

Table VI: Generation of recombinant Ad35 viruses on the new established complementing cell line clone #45.

	GFP Expression				x
	Day1	Day3	Day 6	Day 8	
Transfected constructs					
pAdApt35.eGFP + pWE.Ad35.pIX-rITR	15%	20%	30%	50%	
pAdApt35.eGFP + pWE.Ad35.pIX-rITRΔE3	10%	25%	40-50%	100%	
pAdApt5.eGFP+ pWE.Ad5.AflII-rITR	15%	25%	20%	20%	
untransfected	0%	0%	0%	0%	
	CPE events				x
	Day1	Day3	Day 6	Day 8	
Transfected constructs					
pAdApt35.eGFP + pWE.Ad35.pIX-rITR	0	0	1	several	
pAdApt35.eGFP + pWE.Ad35.pIX- rITRΔE3	0	0	several	80%	
pAdApt5.eGFP+ pWE.Ad5.AflII-rITR	0	0	0	0	
untransfected	0	0	0	0	

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